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Volume 20

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Contents

Subject index

Author index

ABSTRACTS

13th MEETING OF THE HUNGARIAN BIOPHYSICAL SOCIETY

3 - 5 July, 1985

DEBRECEN

C O N T E N T S

CYTOMETRY AND MUSCLE

CYCLOTRON

PHYSICAL EFFECTS AND METHODS IN AGRICULTURE

MEMBRANE

MISCELLANEOUS

APPLICATION OF FLOW CYTOMETRY IN PLANT IMPROVEMENT

L. Trón[1], A. Mészáros[2], M. Balázs[1], J. Szöllősi[1]

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Recent developments and advances in plant physiology /regeneration of the whole plant from a single protoplast/ made flow cytometry a promising method of practical importance in the field of plant improvement and hybrid plant production. Protoplast fusion seems to play a role of growing significance because of the wide range of opportunities offered by this technique for transfer of genetic information.

Application of protoplast fusion aiming hybrid plant production of species with outstanding economic value is limited by the inherent labor-intensive features of the method. Flow cytometry provides an universal possibility to solve the problem of distinguishing and physically separating heterocaryons from homocaryons and unfused protoplasts in a reliable and high speed manner. The basis of the method lies in the vital staining of protoplasts with different fluorescence markers and the gating of sorting by the simultaneous appearance of fluorescence emission from both of the parental sides.

A further potential application of flow sorting may be based on intrinsic fluorescence of certain protoplasts or specific staining for well defined cellular constituents.

FLOW CYTOMETRIC DETERMINATION OF SPERM NUMBER IN DILUTED BULL EJACULATES

L. Mátyus[1], T. Takács[2], J. Szöllősi[1], M. Balázs[1], R. Gáspár jr.[1], G. Szabó jr.[1], L. Trón[1], I. Resli[2], S. Damjanovich[1]

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High-speed flow cytometric investigations were carried out to determine objectively the number of spermatozoa in different semen samples. Analysis was made with a FACS III /Becton-Dickinson/ fluorescence-activated cell sorter and separator, at a speed of 1.000 cells per a second.

It was observed that the number of cells counted on the basis of light-scattering may considerably differ from the actual data as a result of same-sized particles present in the sample. The total number of cells calculated as the sum of live /FDA^+/ and dead /PI^+/ cells in a double stained sample is not correct, since a certain part of the sperm population is between the two specified states and does not take up either of the dyes. The number of spermatozoa can be determined exactly if the sample is treated with Nonidet P-40 detergent and stained with propidium iodide. All the cell nuclei can be detected in this way and the actual number of spermatozoa in the sample can be determined with a relative error of 2 to 5 percent.

COMPARATIVE INVESTIGATION OF MEMBRANE POTENTIAL MEASUREMENTS BY FLOW CYTOMETRIC AND SPECTROFLUORIMETRIC METHODS

M. Balázs, L. Mátyus, J. Szöllősi, L. Trón, S. Damjanovich

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Cytoplasmic membrane potential of BALB/c mouse thymocytes has been determined by flow cytometric and spectrofluorimetric methods, using 3,3'-dihexyl-oxacarbocyanine /$DiOC_6$/ fluorescent dye. The amount of this lipophylic cation incorporated into the cytoplasmic membrane is dependent, among others, upon the transmembrane potential, so the dye is suitable for continuous monitoring of changes in value of this parameter.

Results of the flow cytometric and spectrofluorimetric methods obtained with aliquots of the same stained samples has been compared. A time- and concentration-dependent depolarizing effect of the dye $DiOC_6$ has been found. This dye-induced depolarization can be observed even at very low /order of 1-10 nM/ concentrations, although after prolonged incubation time.

DETERMINATION OF THE EFFICIENCY OF FLUORESCENCE RESONANCE ENERGY TRANSFER ON NORMAL AND LEUKEMIC MOUSE LYMPHOCYTES BY THE USE OF A SINGLE-BEAMED FLOW CYTOMETER

J. Szöllősi[1], L. Mátyus[1], L. Trón[1], M. Balázs[1], I. Ember[2], S. Damjanovich[1]

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Recently, we have developed a method for the determination of the efficiency of fluorescence resonance energy transfer /FRET/ on a single cell basis by the use of a flow cytometer capable of dual wavelength excitation /Trón, L. et al., Biophys. J. 45: 939-946, 1984/. With some modifications, the method has been applied successfully for a single-beamed flow cytometer /FACS III/. The efficiencies of FRET were measured between fluorescein and rhodaminated concanavalin A /Con-A/ bound to normal and leukemic mouse lymphocytes. There was no difference between the two cell types in FRET efficiencies determined at different fluorescein to rhodamine molar ratios. Fitting the values of FRET efficiencies to theoretical curves describing energy transfer processes in two dimensions, an absolute surface density of $/1\pm0.15/x10^4/\mu m^2$ can be determined for Con-A binding sites. However, the smooth sphere model leads to calculated surface densities of $3,6x10^3/\mu m^2$ and $2,9x10^3/\mu m^2$ for normal and leukemic lymphocytes, respectively. These data confirm our earlier finding, that the Con-A binding sites on the cell surface are, to some degree, microaggregated.

FLOW CYTOMETRIC IDENTIFICATION OF LIVE AND DEAD SPERM SUBPOPULATIONS IN BULL EJACULATES

J. Szöllősi[1], T. Takács[2], M. Balázs[1], R. Gáspár jr.[1], L. Mátyus[1], G. Szabó jr.[1], L. Trón[1], I. Resli[2], S. Damjanovich[1]

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Flow cytometry was used to examine the distribution of dead and live cells in fresh, equilibrated and deep-frozen bull sperm samples.

Fluorescein diacetate /FDA/ proved to be a reliable means of measuring live cell fraction only in the case of fresh samples. FDA^+ /live/ subpopulation does not detach from the rest of the population in equilibrated samples and the overlap of FDA^+ and FDA^- subsets is even more marked in frozen samples. These discrepancies between the results obtained at fresh and frozen or equilibrated samples are probably due to both glycerine and egg yolk content, as well as to the cellular changes caused by freezing in non-fresh samples.

$DiOC_6$ has been found to be apt to mark the living cell concentration in deep-frozen and equilibrated samples. The fluorescence intensity of $DiOC_6$ correlates with the membrane potential. This allows us to distinguish between the different subpopulations of live, functionally dead, and totally dead cells easily.

FLOW CYTOMETRIC CONTROL OF BULL SEMEN EQUILIBRATION BEFORE FREEZING

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One of the most important phases of semen preservation in the deep freezing technology is the equilibration /storage at $4^{\circ}C$/, the duration of which effects the post-freezing viability of spermatozoa. In order to decrease the proportion of damaged cells it is very important to optimize the equilibration time. Sperm cells of the same ejaculates were handled simultaneously with TRIS and Jondet extender. Every second hour from the 2nd to the 30th hours of equilibration aliquots were frozen by the Cassou method. The deep frozen straws were kept in liquid nitrogen. Thawn samples were diluted 1:100 with RPMI buffer and stained with fluorescent dyes, dihexyl--oxacarbocyanin iodide /$DiOC_6$/ and propidium iodide /PI/.

Stained samples were analysed using a flow cytofluorimeter, and the proportions of live /$DiOC_6^+$/ and dead cells /PI^+/ were determined as a function of the equilibration time. Optimal live fractions were obtained at different duration of equilibration using TRIS and Jondet extender.

COMPUTER ANALYSIS OF RESULTS GAINED BY FLOW CYTOMETRY

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Multiparameter analysis of heterogeneous cell populations can be performed by flow cytofluorimetry on a cell by cell basis with high speed and accuracy. It is convenient to fit recorded spectra of homogeneous cell populations, usually collected in 128 data channels, by single Gaussian functions. Data originating from a heterogeneous cell population can be described as a sum of individual Gaussians, each of them characterizing a subpopulation within the whole sample. It is desirable to resolve the overlapping Gaussian functions of such a cell population into component peaks. A specially oriented computer program has been created for this purpose. Using this program one can decompose a combined function into four Gaussians. After an initial guess of the parameters of the composit Gaussian functions several iterations are performed and the calculated and experimental curves are continuously displayed on a CRT.

BIOPHYSICAL ANALYSIS OF DEEP-FROZEN BULL EJACULATES OF HUNGARIAN AND FOREIGN ORIGIN

M. Balázs[1], T. Takács[2], J. Szöllősi[1], R. Gáspár jr.[1], L. Mátyus[1], G. Szabó jr.[1], L. Trón[1], I. Resli[2], S. Damjanovich[1]

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Traditional laboratory methods /cell counting with hemacytometer and microscopic estimation of motile spermatozoa fraction/ and flow cytometry was used for a comparative analysis of 21 deep-frozen semen samples. Nine imported samples /different in origin/ and 12 samples obtained from 4 Hungarian animal breeding stations were analysed throughout the experiments. The results showed no difference between the averaged sperm number and live cell proportion gained by traditional and flow cytometric methods. At the same time, there were remarkable discrepancies between the results by these methods in the case of individual samples. The reproducibility of flow cytometric measurements /CV=2-5 %/ exceeded significantly that of conventional laboratory measurements /CV= 25 %/. Flow cytometry also gave evidence of the approximately identical total cell number in all the analysed insemination doses. However, the live cell fraction was significantly /$p<0.05$/ lower in imported samples than in Hungarian ones.

SENSITIVE FLUORESCENCE METHOD FOR THE CHARACTERIZATION OF VITALITY OF CELLS

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Dye exclusion tests are widely applied to measure vitality of cells. These methods rely on the observation that appropriate dyes /e.g. propidium iodide - PI/ can only penetrate through damaged membranes. The fluorescein diacetate /FDA/ test also can be used for the same purpose, as the fluorescein, the hydrolysed product of the FDA is accumulated only in cells with intact cytoplasma membrane.

The essential drawback of the above procedures lies in the fact that the sum of the live /FDA^+/ and dead /PI^+/ cells is less than the total number of the cells. According to out method cellular vitality can be investigated by staining with dihexyloxacarbocyanine iodide /$DiOC_6$/. It is possible to determine with this membrane potential indicator the fraction of cells with physiological membrane potential as well as that of cells having decreased or zero membrane potentials because of membrane damage. In our experiments aliquots were taken from a cell suspension after different incubation time at 45^oC for simultaneous PI, FDA and $DiOC_6$ staining. The results show that the $DiOC_6$ test is superior to the others in yielding more reliable and detailed information about cellular vitality.

DENSITY AND MODULATION OF SURFACE ANTIGENS ON MONONUCLEAR CELLS IN SLE

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In systemic lupus erythematosus /SLE/ many immunological abnormalities have been observed. Normal functions of lymphocytes require antigen binding receptors /sIgM on B lymphocytes and T3-Ti complex on T cells/ and surface molecules for cell-cell interactions /T4, T8 and HLA-Dr./. In this study the surface density of these antigens was measured with immunofluorescent staining by the use of a flow cytometer on mononuclear cells from patients with SLE. The densities of T3, T4, T8 and sIgM were the same on SLE lymphocytes as on lymphocytes from healthy subjects, while the density of HLA-Dr antigens decreased on the SLE patients' monocytes.

The modulation of T3-Ti complex is an obligatory step in the physiological T cell activation. This membrane process was also studied in patients with SLE. The modulation of T3 antigen after culturing the cells in the presence of anti-T3 monoclonal antibody was measured as the antigen density described previously. The modulation of T3 antigen was impaired in the disease, this defect might result in the known T cell hypofunction. There was no alteration in the modulation of T4 and T8 antigens.

THE INVESTIGATION OF THE ACTIVATION HEAT IN VARIOUS CROSS-STRIATED FROG MUSCLES

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According to the original definition of Hill /1949/ the activation heat is produced by a trigger reaction during which the muscle gets to such position in which it can shorten and work but its contractile component makes neither inner nor outer shortening.

Its determination was made by the subtraction of the shortening heat from the current heat production. The activation heat can extend from 10 to 80 % of the total heat production according to the results obtained by different methods.

Splitting of cross-bridges without macroscopic shortening may be one explanation for these different results.

Our measurements were made so that the muscle was stretched to "Δ-state" where the excitation-contraction coupling exists but the myofibrillar overlap is zero. Experiments were made in m. sartorius, m. semimembranosus and m. gastrocnemius of Rana esculenta at room temperature during single twitch /stimulated with square wave impulse of 6 V, 30 ms/.

According to our measurements and the latest data in literature the activation heat is about one third of the total heat production. The maintenance heat in tetani of 2 s 66 Hz is about 20 % of total heat.

THE EFFECTS OF ACTIN ON THE POSITION OF THE MYOSIN HEADS IN THE INSECT FLIGHT MUSCLE

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The molecular mechanism of muscle contraction is supposed to consist of cyclic movement of myosin heads. During a cycle the interaction of the myosin heads with the actin filaments results in the contractile force. Two states of this cycle were visualized by electronmicroscope: in rigor the heads were attached to the actin filaments in angled position, whereas in relaxed state they were dissociated from the actin filaments and arranged in perpendicular position. The studies which dealt with the head movement were carried out mainly in the presence of actin filaments. This electronmicroscopic study tries to answer the question how the actin molecules influence the positions of the myosin heads. The fresh muscles were chemically skinned with TRITON 100 X nonionic detergent and treated with either relaxing or rigor inducing solutions.

Relaxed muscle: In the H zone the myosin heads are dissociated from the filament backbone and dispersed among the myosin filaments.

Rigor muscle: In the absence of actin the myosin heads are adhered to the filament backbone and become invisible. In the presence of actin /either in filamentous form or in cross-linked G form/ the myosin heads are swinged out from the backbone and become visible.

Conclusions: In contrast with the generally accepted opinion the heads not attached to actin filaments can also be visualized. In rigor muscle two states of the myosin heads exist: in the presence of actin the heads dissociate from the backbone /independently whether they form crossbridges or not/, but in the absence of actin the heads are associated with the filament backbone.

DYNAMICAL ORDER OF CROSS-BRIDGES IN THE PRESENCE OF ATP AS STUDIED BY SATURATION TRANSFER EPR

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Cross-bridge ordering and motional dynamics of the myosin head region were investigated in glycerinated skeletal and cardiac muscle fibres using maleimide nitroxide selectively attached to the fast reacting thiol sites in myosin.

The EPR spectra of muscle fibres which were spin-labelled after dithionitrobenzene-EDTA treatment exhibited large orientational order. The ordering of cross-bridges depended strongly on the mechanical state of the muscle fibre. In relaxing medium and in activating solution the cross-bridge order was significantly decreased in comparison with rigor showing that in relaxed state and during the force generation cycle the steric distribution of myosin heads with respect to the fibre axis was strongly influenced by ATP.

The disorder is dynamical on the saturation transfer /ST/ EPR time scale; the rotational correlation times were decreased from $\tau_2 > 1$ ms to about 200 μs and 50 μs, respectively, providing evidence that the motion of the myosin domains was anisotropic in the presence of ATP.

The comparison of the conventional and ST EPR spectra for skeletal and cardiac muscle fibres showed that the smaller ordering in cardiac muscle even in rigor was partly due to residual motion of myosin heads.

EFFECTS OF LEG-IMMOBILIZATION ON THE PARAMETERS OF MUSCLE MECHANOGRAM

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Adaptation of soleus muscle from New Zeland rabbits /2-2.5 kg/ was studied following 0, 1, 2, 4, 6 weeks immobilization by plaster cast. Our aim was to find out changes of the mechanical parameters induced by electrical stimulation of the soleus after different periods of immobilization.

The intact muscles were removed quickly /< 5 min/ from anaesthetized animals and placed immediately into Tyrode solution. The solution was thermostated and bubbled with oxygen.

Isometric contractions were evoked by single stimuli. The stimulus was square pulse of voltage and it was standardized /100 mA and 10 ms/. These parameters of stimulation provided supramaximal stimulus at 50 mN pre-tension. The mechanical responses were analyzed by a microcomputer.

Results: with increasing the time of immobilization the half-time of contraction decreased from 70 ms to 58 ms on average /Sy=15%/. The maximal tension normalized for the mass of muscle was increased from 200 mN/g to 600 mN/g. The rate of contraction rised from 4 N/sg to 24 N/sg the rate of relaxation rised from 3 N/sg to 9.5 N/sg /$\frac{dF}{dt}$/. The impulse of twitch increased from 6 mNs/g to 36 mNs/g.

Our results suggest that the slow fibres of soleus turned into fast ones in a high proportion. This conclusion is proved by histochemical and biochemical experiments, as well, performed at our institute.

KINETIC INVESTIGATION OF K^+ EFFLUX IN MUSCLE

Z. Hummel

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Frog sartorius muscles were pretreated in 11.6 M /87%/ ion-free glycerol for one hour at $+2^{o}C$. Then half of them were put in 5.8 M glycerol solution containing 55 mM NaCl at $-12^{o}C$. The release of potassium during exposure to the 5.8 M glycerol solution was significantly faster in the presence of Na^+ than in ion-free circumstances. The measured K^+ content of muscle as function of time of the 5.8 M glycerol treatment was approximated by sum of two exponential terms with different time constants, namely as

$$K(t) = A \cdot \exp(Bt) + C \cdot \exp(Dt).$$

The experimental K^+ contents fit very well this type of theoretical curve. Two fractions of potassium were found. The fast fraction, with 7×10^{-1} ($hour^{-1}$) of time constant, got in the glycerol space at the very early beginning of the 5.8 M glycerol treatment, but the other fraction, with 9×10^{-3} ($hour^{-1}$), got to the glycerol space very slowly. The fast K^+ fraction of the muscle treated with Na^+ containing glycerol was 0.43 mgK/g wet weight larger than that of the muscle in ion-free glycerol. Indirect evidences for existance of a Na^+ binding reaction were found at the outset of glycerol exposure of the muscle. The Na^+ binding induces the fast K^+ release. On the basis of the Na^+ binding model, the propagated action potential is interpreted.

THE IMPORTANCE OF BIOPHYSICAL METHODS IN THE PRACTICE OF ARTIFICIAL INSEMINATION

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The correct characterization of semen is of great importance in artificial insemination material production. The objective biophysical determination of quantitative and qualitative parameters of semen may rationalize sperm production of an artificial insemination station. The number of offsprings of bulls of the highest genetic value can be increased in this way.

Recently used routine methods of semen qualification are not satisfactory for objective evaluation. Flow cytometry, as a new method of semen evaluation, offers a fair possibility to determine the exact number of sperm cells and the percentage of living spermatozoa in both freshly taken and deep-frozen samples. Exploring the advantages of this method, the number of straws made from one ejaculate can be increased and ejaculates with low fertility can be distinguished. The method can be used also to optimize semen preservation techniques.

Flow cytometric detection of X and Y chromosome bearing spermatozoa offers immediate control for sex orientation methods by various physical or biochemical techniques.

THE APPLICATION POSSIBILITIES OF THE APPARATUS MUTACALC

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During the operation of the apparatus MUTACALC controlled phage development cycles take place. In the case of each cycle the parameters characterizing the cycle can be determined: the latency period and its standard deviation, the multiplicity of infection and in some cases the magnitude of the average burst size as well.

The biological process characterized with the parameters can be influenced with physical and chemical effects at the following points of attack:

- on the host bacteria
- on the phages
- on the phage-bacterium complexes

According to our measurements a relation can be shown between the degree of the effect and the change of one or more parameters of the phage-development cycle. The most important biological reactions, which can be characterized quantitatively: the kinetics of phage inactivation; - the toxicity /which can be measured by the increase of the latency period of the average burst size of the bacteria/; the repairable or non-repairable nucleoprotein damages and their distinction. By the determination of these effects and parameters the phage development cycle can be optimized as well.

The listed effects may appear jointly, therefore their appropriate separation is necessary at each measurement. In our opinion the solution lies in the complex computer process control and data processing /expert system/ connected to the apparatus /under development/.

MEASUREMENT OF TOXIC EFFECT OF CHEMICALS WITH THE APPARATUS MUTACALC

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By the aid of the apparatus MUTACALC the various points of attack of the toxic effects of chemicals can be investigated. The effect is characterized quantitatively in each case. The possibilities of measurement are the following:

- The toxic effect exerted directly on phages results in inactivation kinetics of multi-hit character. In this case the effect is characterized by the kinetical constant belonging to the different concentrations of the chemical.

- The growth rate of bacteria is decreased by toxic chemicals. In this case the characteristic quantity is the decrease of the growth rate related to unit chemical concentration.

- In phage-bacterium complexes the latency period of phage development increases compared to that of normal phage development. The relative increase /related also to unit concentration of chemical/ gives in this case the degree of toxicity.

- The direct effect on phages in the course of intrabacterial development can be distinguished from the effect appearing on bacteria. The growth rate of bacteria is set by the variable dilution rate to the value corresponding to the toxic value. The latency period measured in this way are compared with the degree of toxicity measured on the phage-bacterium complexes.

The cases are illustrated by concrete examples which can be measured with the apparatus MUTACALC /in construction/.

MUTACALC APPARATUS TYPE OP-217 FOR DETERMINATION OF MUTAGENIC ACTIVITY OF CHEMICALS

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The mutagenic activity of chemicals is determined via the continuous evaluation of the phage development cycle taking place within a given phage-bacterium complex /T7, E. coli B/. By means of the apparatus, the quantity characterizing the mutagenic activity of chemicals, the so called dimer equivalent dose /DED/ can be determined. The host cells of stable physiological parameters are produced in the chemostat at constant temperature and with continuous stirring and oxygen supply. The division time of bacteria can be set by the rate of nutrient influx. In the treatment unit the phages are incubated at constant temperature in the presence of the chemical to be tested. The apparatus feeds host cells from the chemostat and appropriately diluted and treated phages from the treatment unit into the biological reactor. After the reaction the sample is forwarded from the reaction cells to the flow-through cuvette of the optical measuring unit. The data characterizing the inactivation of phages are obtained from the light scattering of the solutions which are further evaluated by the data processing unit of the microprocessor unit. The results obtained can be visualized on a CRT display and/or for further processing a hardcopy can be made.

COMPARISON OF ACTION SPECTRA OF CRYSTALLIZED URACIL LAYERS AND T7 PHAGES BY THE AID OF MUTACALC

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Upon illuminating a crystallized thin uracil layer by an adequate UV-light, a process of dimer formation starts. This process results in a decrease of layer's absorption, which was applied to construct an UV-dosimeter. Since the spectral sensitivity of the uracil layer and the T7-phage /considering it as a chromosome model/ are in the same range of wavelength, the uracil layer could be suitable for measuring the genetical damaging effect of UV-light.

The action spectrum of T7-phage with respect to its inactivation was determined by MUTACALC equipment. This means that we could define the genetical damaging effect of UV-light as well.

Dividing the determined action spectrum of uracil layer due to the decrease of its absorption caused by the UV-light, by the action spectrum of T7-phage a special function was obtained. This function determines the characteristic features of the filter used to correct the illuminating light for our dosimeter with the aim of getting an average effect proportional to the genetical damaging effect of the original illuminating light.

Thus the thin layer with the filter is suitable for measuring selectively the damaging effect of very low intensity radiation forming a component of composite light /for example sunlight/.

MUTAGENECITY TESTING OF FOOD CONSTITUENTS BY THE APPARATUS MUTACALC

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A new automated in vitro screening system and apparatus, MUTACALC was developed for the characterization of mutagenic effect of chemicals. The method is based on the measurement of the inactivation curve of T7 phages with the aid of E. coli B host cells. The mutagenicity index /DED/ is calculated from the initial part of the dose-effect curve.

Up to now about 60 chemicals have been investigated, among others several psoralen derivatives. Psoralens are present in many plant species including parsley, carrot, celery etc., their binding to nucleoproteins takes place in two steps. The first step is the formation of a radiation independent complex /dark binding/, the second step is induced by irradiation with UV-A radiation. Both steps were investigated by MUTACALC. Besides, other foodstuffs were tested: artificial sweeteners as sodium saccharin and sodium cyclamate, sodium nitrite used for meat preservation and agents which improve feed conversion of animals: Ralgro, Carbadox, Zeranol, 17-β-estradiol etc. Based on the obtained mutagenecity index /DED/ the chemicals can be classified according to their mutagenic potency. The less the DED value the more dangerous the chemical is with respect to mutagenecity. Our results are compared with the results of other qualitative mutagenecity/carcinogenecity tests.

SPERM ENZYME ACTIVITY AND LECTIN-BINDING MAY BE USED FOR DETECTION OF AGENTS WHICH HARM MAMMALIAN GERM CELLS

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Sperm is readily available for the study of environmentally-induced damage to germ cells. We tested the hypothesis wether or not agents known by other methods to damage germ cells can be detected by changes in the histochemical single sperm enzyme activity and lectin-binding assays that others or we developed.

- When mouse sperm were assayed for acrosin, alpha-glycerol-phosphate or succininic dehydrogenase activity following intraperitoneal /ip/ or inhalation /ih/ exposure, the mutagens ethylnitrosourea /ENU; ip/ decreased the activity of the 3 enzymes, whereas ethylmethanesulfonate /ip/ and benzene /ih/ decreased the activity of 2 enzymes. The teratogen hydroxyurea /ip/ and the reproductively toxic agent dimethylmethylphosphate /DMMP; ip/ decreased the activity of 2 enzymes. Ethylene dibromide, suspected of causing infertility in men, and DMMP, both given ih, decreased the activity of 1 enzyme.

- Only a small fraction of mouse sperm binds the lectins BAN and UEA. In one experiment 5.3, 18 and 312 X 10^{-5} sperm bound BAN in saline controls and in mitomycin C /MC/ or ENU-treated mice, respectively. UEA bound to 1.9 and 22 X 10^{-5} sperm in controls and in MC-treated mice. - We found that the enzyme activity and lectin-binding assays may be used for detection of agents which damage germ cells. - This research was supported by grants from the Faculty Research and Creative Activities Fund of Western Michigan University.

PLASMID MEDIATED RESISTANCE TO IONIZING IRRADIATION IN ESCHERICHIA COLI

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The N group R plasmid R46 /R-Brighton. R1818, TP120/ and its derivative pKM101 have been shown to increase spontaneous mutagenesis, susceptibility to UV induced mutations and to reduce susceptibility to killing by UV.

In our experiments the ^{60}Co-gamma sensitivity modifying effect of the above two drug resistance plasmids was studied. R46 R-factor increased the survival of the wild type strain and had no effect on the survival of recA$^-$ and recA$^-$recB$^-$ double mutants. A well detectable increase in ^{60}Co-resistance was also found in recB$^-$ mutant harbouring R46 R-factor. 1 mg/ml of 5-fluorouracil eliminated R46 R-factor from the parent and its rec$^-$ mutant strains. recB$^-$ /R46/ lost not only the antibiotic resistance coded by R46 R-factor but also its radioresistance. pKM101 plasmid also enhanced the bacterial survival in the wild type strain, in its recB$^-$recC$^-$ and in recF$^-$ mutants but had no effect on the survival of recA$^-$ mutant.

Thus the effects proved to be dependent on recA$^+$ genotype but not on recB$^+$, recB$^+$recC$^+$ and on recF$^+$ genotypes. The recA$^+$ and lexA$^+$ genes are known to control the inducible DNA repair and the events are collectively termed as SOS responses. It is most probable that R46 and pKM101 specially enhance the ability of the cell to carry out SOS processing.

RADIO-MUTATION EXPERIMENTS OF SUNFLOWER /HELIANTUS ANNUUS/

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Widening the basic material for purification is important with respect of the successful development in the classical and the heterosis purification. One of the possible ways of this is the irradiation of sunflower seed-corn with fast neutrons which we have done at the Atomic Research Institute, Debrecen.

According to our investigations, in the case of those sunflower species which are oleaginous to a great extent, mortality occurred at relatively low fast neutron doses. The species showed different sensitivities /in the case of GK 70 type 50 Gy, and Sz-1 type above 16 Gy caused mortality/. The phytoparametres which were important with respect of the yield, were changed by the fast neutron irradiation in different rates and directions in different species. Generally it can be stated that increasing doses decreased both the achenium numbers and achenium-weights of sunflowers /except in the case of GK 70 type in which a stimulative influence was seen at 1.3 Gy/. The total achenium-weight and chell-weight characteristic at a thousand achenium increased at both species, otherwise in seed-weight at GK 70 there was a decrease and at Sz-1 an increase.

Our investigations proved that the fast neutron irradiation of a species did not result in adventageous changes as regards purification. In spite of this, it is important to investigate the mutations of genetically homogeneous lines produced by biophysical ways.

FAST NEUTRON TREATMENT OF SOYBEAN SEED-CORN FOR MORE GENERATION

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In our investigations aiming at increasing the protein content of plants, we tried to raise the genetical productivity of soybean by the way of biophysics.

For the sake of complex increase of crop potential, fast neutron treatment was performed on Merit Canadian and S-1346 American soybean species at the Atomic Research Institute, Debrecen. Selection of the mutants with positive purification values began in the M_2 generation and the evaluation of the offspring, as it is usual in the case of self-fertilizing plants, was connected with individual selection /pedigree growing/.

According to our experiments, in soybeans of high oil-contents irradiation greater than 120-130 Gy dose was lethal and the S-1346 species was more sensitive to the irradiation. Lower value than the 50 per cent of the control plant-height did not occur in our treatments since the irradiation dose prevented even the formation of primordial leaves.
In case of irradiation in the M_1 generation we observed the least pods,the smallest plant height and stem thickness up to 60 Gy dose. From the factors describing the crop the number of pods by plants, the number of seeds, the seedweight and seed number by pods decreased with increasing the dose. On the other hand, the seed length and breadth, and the total mass of a thousand seeds were in positive correlation with the dose. /The last showed 20-30 g increase as compared to the not processed seeds/. In the case of lower doses the shortening of soybean breeding season, while with greater doses its prolongation was experienced.

The individual selection for higher seed mass was parallel with a decreased plant height and leaf surface but the assimilative achievement increased. The starting popula-

tion had a greater variety of shapes; now we had low, thick--set, bushily branching off, thick stem shapes. In the plant mass the ratio of useful crop increased /in the M_3 generation 0.45 Harvest-index lines/.

In M_3 generation we succeded in the selection of such soybean lines which produced 50-60 % greater seed mass than those not treated. These would be adequate partners in cross-fertilization to increase the productivity of soybean.

USE OF IRRADIATION INDUCED MAIZE MUTATION LINES IN PLANT IMPROVEMENT

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Mutation populations with greater varieties play an important part in the improvement of maize cultivation biological bases. From them hybrids suitable for the current demands can be produced by up-to-date proceedings.

We studied the purification value and use of the genetically secure mutation lines with different morphological characters.

The oeco-stableness investigations showed that the hybrid combinations produced by mutation lines can be cultivated with a greater safety than the standards. Maize ripening earlier can be produced by mutation lines used as males and the nutriment accumulating period of the maize seed can be lengthenes. The water-giving ability of the hybrid-combinations can be improved by mutation lines, as well.

The best productivity can be reached by those mutation lines which were cross-fertilized with single-crossed mothers produced by inbred brunch. The productive capacity of maize genotype, the individual biomass production, and the Harvest--index can be increased in the mutation-combinations.

Mutation lines show great specific combining ability regarding the dry-mass production which determines the silo value, while they have a high general combining ability value in the cob-number important in the view of seed-crop.

We can improve the protein, macro- and micro-element contents of the different plant parts and the corn of maize by using mutation lines. The inside content components can be improved, too, in the mutation combinations by increasing the crop.

EFFECT OF GAMMA RAYS ON THE VARIABILITY OF MAIZE

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For breeding varieties of favourable genetic structure, there is a need for increasing the amount of breeding material and enriching gene reserves. In the late 1950s we treated the pollen of a Transsylvanian strain of maize with 15 Gy ^{60}Co gamma irradiation. This resulted in mutants of diverse morphological characters, of which several types of mutant lines were selected. Some of the isolated mutants were highly productive types producing 4-5 or, occasionally, over 10 ears per plant. In addition to this, bushy and leafy types, types with upright leaves, as well as narrow leaved so called cron-grass types were isolated. Compared to standard varieties, normal in-bred x corn-grass hybrids yielded more green and dry matter and the ratio of the various plant parts as well as their chemical composition /protein content, the content of macro- and microelements/ improved.

In the experiments it was noted that certain forms became stable. Crossed with normal maize lines, these yielded hybrids with good characters. Other mutant forms, however, did not become stable and new forms kept appearing despite long periods of self-pollination. Recent mutant maize population is still producing new forms, although forms that are easy to stabilize also crop up as a result of self-pollination. Various hybrid combinations were produced form the already stabilized mutant lines.

Results of several years of experimentig have proved, that by using mutants, the ratio of the various plant parts /leaf vs. stalk vs. ear/ can be improved, which is especially useful in breeding silage type maize. Standability as well as dry matter content and yield could be increased. Chemical composition, physiological as well as other characters of economic importance could be improved.

EFFECT OF FAST NEUTRONS ON THE MUTABILITY OF SOYBEAN

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In the Atomic Research Institute of the Hungarian Academy of Sciences in the years 1980 and 1981 we treated MERIT /Canadia/, S-1364 /USA/, and ISz-15 /Hungarian/ soybean varieties with 3.23 - 174 Gy fast neutrons. During the years, several types of mutant lines of the above variaties were selected, the best of which was studied in small parcel control experiments. The results are summed up as follows.

In the year of the treatment the radicles were damaged by greater dosage of irradiation. These plants developed only while nutrients in the seedlobes were insured. Later they died.

The plant heights were smaller if the soybean was treated with a greater dosage in the year of the treatment, at the same time the use of a smaller dosage resulted in stimulation of growth. In the later mutant generations a close positive correlation can be noticed between the dosage and the branching.

In the case of selected mutant lines, as regards the number of seaths and seeds per plant, certain dosages /18.2, 22 and 54.5 Gy/ had a favourable effect. As compared to the control, the number of seeds and seaths increased. Certain mutant lines gave outstanding values as regards the protein content /47-48 per cent/. The oil content also showed an increase.

THE EFFECT OF FAST NEUTRONS ON LUPINUS MUTALIS

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We have been doing experiments for induced mutation from 1982. The following species were involved: Lupinus albus, Lupinus luteus, Lupinus angustifolius and, from 1984, the Lupinus mutabilis, too.

This species has high protein content and high oil content, too. The irradiation of the dry seeds was performed at the 150 KV neutron generator of the Institute of Nuclear Research of the Hungarian Academy of Sciences in Debrecen. We produced monoenergetic neutrons of 14.6 MeV through D+T reactions. The yield was about 10^{10} neutron/s.

On the first day after irradiation we sowed the seeds in field by hand. On the sixth week after sowing we measured the height of the hypocotil, the number of leaves, the length of the stalk of the leaves and the length of all leaflets.

We determined the mean, the variance, and the standard deviation of the measured values. We found that 40-50 Gy /in CH equivalent absorbed dose from the neutron flux/ dose caused less than 50 % of seedling growth reduction in M_1 generation.

This year we have sown the M_2 generation for investigation.

EFFECT OF THERMAL NEUTRON ENERGY ABSORBED BY SEED TISSUES, LINKED WITH BORON CONTENT, AT THE ONSET OF GROWTH OF PEA AND ON ITS SEVERAL BIOCHEMICAL CHARACTERS

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For more reproducible plant physiological responses to ionizing radiation, /according to our opinion/, it is essential that the plant's responses should be investigated on the basis of energy actually absorbed by the plants. This trigger radiation absorption process is influenced mainly by the type of irradiation, the energy spectrum, fluxes, geometry of irradiation, and elemental composition, as well as the physiological state of plants. To demonstrate this, calculations were performed on the thermal neutron /2.53×10^{-2} eV/ energy absorption of pea seeds by means of a "five element" /C,O,H,N,B/ tissue method, using appropriate kerma factors. The original elemental composition of seeds was altered by soaking a certain number of the seeds in boron solution of 1000 ppm concentration for 24 h /boron has a 760 barn activation cross section/. Therefore, in pea seeds enriched or not in boron, exposure to the same neutron flux of 1.7×10^{6} $n.cm^{-2}s^{-1}$ resulted in two distinct tissue energy absorbances. In our experiments the tissue dose range of 10^{-4} to 10^{-2} Gy was used.

As a consequence of neutron irradiation, the length of roots and shoots was modified - a phenomenon that was linked with larger fresh mass yield. An additive effect between boron as a microelement and the absorbed neutron energy on the respiration of germinating seeds was observed. The activity of α--amylase was accelerated; the photosynthetic pigment contents, e.g. chlorophyll *a* and *b* plus carotinoid compounds, were sensitively influenced by irradiation, a result which enables the optimal absorbed energy of thermal neutrons to be assessed according to changes in the pigment content under the effect of irradiation.

INVESTIGATION ON RADIATION-SENSITIVITY OF DIPLOID AND TETRAPLOID SUDANGRASSES BY NEUTRON IRRADIATION

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During the period 1982-83 studies were conducted with the aim to specify the radiation sensitivity /mainly towards neutron irradiation/ of diploid sudangrass /S. sudanense/ and tetraploid Sorghum almum as well as tetraploid Sorghum sudanense /4n/ produced by colchicin.

The neutrons were generated through the $H^3/H^2,n/^4He$ reaction. The average neutron energy was 14.7 ± 0.2 MeV. The total yield of the generator was about 10^{10} n/sec. The seeds were irradiated by different doses: between 65-200 Gy. After treatment the seed was allowed to germinate between filterpapers in four replications at 25 C^o without illumination.

Hither doses of irradiation /200 Gy/ resulted in significant decrease in germination at the tetraploid sudangrasses. The extent of decrease was nearly the same in both cases.

Upon 200 Gy irradiation the germ weight of diploid sudangrass decreased by 22.1 percent. At the same time the germ weight of tetraploid sudangrasses decreased by 44.3 % and 46.3 %, respectively, in case of S. sudanense /4n/ and S. almum /4 n/. The extent of decrease was larger if the shoot weight was considered, it was 25 % in case of diploid, and 56 % and 63 %, respectively, in cases of tetraploid sudangrasses. The decrease in shoot dry matter was 41.3 %, 46.4 % and 33.6 %, respectively.

The tetraploid sudangrass, produced by colchicin, proved to be more sensitive, in all three treatment, for all the characters studied than was the diploid sudangrass.

EFFECT OF GAMMA IRRADIATION ON THE PROTEIN SYNTHESIS OF PEA

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Dry seeds of pea have been irradiated between zero to 1000 Gy doses. Protein synthesis in the one-week-old ethiolated seedlings was examined by five hours labelling with /^{3}H/leucine and fluorography of the labelled proteins after fractionation by sodyum dodecyl sulphate-acrylamide gel electrophoresis. Polyribosomes were also isolated from control and irradiated pea seedlings and the polyribosome patterns were studied by sucrose density gradient centrifugation.

The incorporation of /^{3}H/leucine was 30 percent higher in the irradiated samples than in the unirradiated ones. Comparing the patterns of protein synthesis, the irradiation induced several new bands of proteins, whereas other bands were relatively increased or decreased.

The contents of mono- and polyribosomes were about 35 percent higher in the irradiated seedlings than in the controls. The polyribosome pattern showed that after irradiation the polysomes degraded to some extent and there was a shift of polyribosomes into monoribosomes and subunits. There changes in protein synthesis are very similar those obtained by stress effects in plants.

THE EFFECT OF GAMMA IRRADIATION ON THE FERTILITY OF COMMON CARP /CYPRINUS CARPIO L./ SPERM

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The success of artificial fish propagation is determined by the high fertility rate of the eggs and by the strong survival rate of the fish fry. The method of artificial propagation of fishes, especially Cyprinidae, offers possibilities for different treatments of eggs and sperm before fertilization.

The fertility rate of the common carp /Cyprinus carpio L./ eggs was improved after fertilization with the sperm treated by 3-20 Gy ^{60}Co gamma rays. The best result was obtained with sperm treated by 5 Gy dose of irradiation. As a result of irradiation, the fertility rates of the eggs were 6-27 percent higher than those of untreated controls.

The survival rate of fry was determined under fishpond conditions in the second year. The survival rate of the 5 Gy treated group was 28 percent higher than that of the untreated one.

BIOCHEMICAL AND MORPHOLOGICAL ALTERATIONS IN BRAIN OF NEWBORN MICE AFTER NEUTRON IRRADIATION, IN UTERO

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Irradiation of mice by 0.5 Gy fission neutron in late gestational period led to the death of about half of the newborn animals in three days after birth. Surviving mice exhibited considerably smaller body weights and even smaller brain weights than those of the unirradiated controls /Vogel and Antal: Radiat. Res. 98, 52, 1984/. Histological examinations, performed in 18-day-old embryonic brain 6 hours after irradiation showed nuclear pycnosis. In the cerebellar external granular layer, i.e. in the actively proliferating cells was found the most pronounced damage. These cells were phagocyted by 12 hours /Antal et al.: Int. J. Radiat. Biol. 46, 425, 1984/. The DNA content of whole brain of three-week-old mice irradiated in utero on 18th day of pregnancy showed that the cell number decreased by 40 %. Synthesis of brain proteins was inhibited especially that of histones and of certain non-histones, separated by two-dimensional gel electrophoresis. Regarding the mechanism of protein synthesis, investigations showed that amino-acylation of transfer-RNA decreased significantly. This might explain the impaired protein synthesis. Irradiation of newborn mice with 0.5 Gy fission neutrons caused neither increase of mortality rate nor reduction in brain size nor in synthesis of brain proteins /Radiat. Res., in press/. These phenomena indicate that the decreased protein synthesis in brain is probably in connection with the damage of neuroblast which actively proliferates at the late stage of pregnancy of mice and thus, it is especially sensitive to radiation. These results might confer with data of Japanese bomb survivors re-evaluated by Otake and Schull /Brit. J. Radiol. 57, 409, 1984/. They showed that severe mental

retardation occurred mainly in descendants of women exposed to irradiation between 8-15 weeks of pregnancy, which is the period of rapid proliferation of neuroblasts.

THE EFFECT OF X-IRRADIATION AND HYPERTHERMIA ON P388 TUMOR

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Different modality combinations /surgery, irradiation, hyperthermia, chemotherapy/ supplementing each other have come to the forefront in the therapy of cancer. According to the tendency of studying the mechanism of action, biological and biochemical changes occuring on the effect of X-irradiation and/or in vitro hyperthermia have been studied in P388 leukemic B6D2F1 mice. In combined treatment, when ascites cells were incubated at 43.5°C for 1 hr, transplanted i.p. and mice were whole-body irradiated with 4 Gy at 12 hr after transplantation, the life-span was increased by 70 %. The effect of local X-irradiation was investigated on solid tumor induced by i.m. transplantation of ascites cells. Solid tumor resulted in metastases in the liver and the spleen. No metastases were observed from solid tumors, induced by i.m. transplantation of ascites tumor cells treated with in vitro hyperthermia at 43.5°C for 1 hr. Local 6 Gy X-irradiation on the 1st day post-transplantation resulted in a 33 % increase of life-span of metastasis producing solid tumors while an 80 % life-span prolongation was observed in case of non-metastasing solid tumors induced by hyperthermia treated ascites cells. The quantity of nuclear proteins in ascites tumor cells and the intensity of synthesis were studied by ^{35}S-methionine incorporation and O'Farrell's two-dimensional gel electrophoresis technique. It was stated that the intensity of non-histone protein synthesis significantly decreased following 4 Gy whole-body X-irradiation. The synthesis and the amount of nuclear proteins also decreased of in vitro hyperthermia at 43.5°C, however, some new proteins, specific to heat-treatment, /the so-called "heat shock" proteins/ were detected in the ranges of 30 kD, 55 kD and 70 kD molecular weight.

EFFECT OF CHRONIC TRITIUM EXPOSURE ON THE MUTABILITY AND RADIOSENSITIVITY OF CULTURED CHINESE HAMSTER OVARY CELLS

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The long term effect of tritium beta radiation was studied on Chinese hamster ovary cells maintained continuously for months in medium containing tritium oxide /HTO/ or tritiated thymidine /3HTdR/ at concentration of 3.7 kBq-37 Bq/ml. During the incubation period the induced gene mutations were determined for the hypoxanthine-guanine-phosphoribosyl transferase /HGPRT/ locus, the chromosomal aberrations and sister chromatid exchanges were analyzed by standard methods. Radiosensitivity of cells was expressed as a function of inactivation rate after exposure to X-rays.

According to the experimental data the chronic tritium beta irradiation resulted in a biphasic response in mutations tested. After 1-2 months' exposure to tritium there appeared to be a more effective reparation, while later /3-6 months' exposure/ there developed a hypermutability of cells which was verified by treatment of cells with a chemical mutagen, ethyl methanesulfonate /EMS/. Regarding the radiosensitivity, no differences could be observed as compared with the controls.

RADIOSENSITIVITY OF BONE MARROW FIBROBLAST COLONY-FORMING UNITS AND THEIR GROWTH KINETICS AFTER IRRADIATION

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Murine bone marrow derived fibroblastic colonies /CFU-F/ are regarded as part of the haemopoietic stroma. In order to obtain information on the effect of relatively low dose /0.5 Gy/ gamma irradiation on the CFU-F population, in vitro radiosensitivity of CFU-F was determined and growth kinetics of CFU-F from normal bone marrow or bone marrow from BDF_1 mice irradiated with 0.5 Gy were compared. Since a strict correlation between cell count and surface area of colonies was found, and CFU-F colonies are known to be clones, their growth kinetics was characterized by their surface area. The data obtained indicated heterogeneity of normal CFU-F population: the calculated population doublings in 7 day-old colonies varied from 5.9 to 8.0, suggesting that the CFU-F population consists of subpopulations with various doubling capacity. The radiosensitivity of these subpopulations proved to be different, too. An in vivo gamma irradiation of 0.5 Gy did not reduce significantly the femoral CFU-F content. The reduction was quickly reversed and a significant overshoot was observed 10-11 weeks after the exposure. Transient changes in the ratio of CFU-F subpopulations and a slightly increased doubling time of CFU-F from 0.5 Gy irradiated mice may be responsible for this late overshoot.

THE EFFECT OF RADIOPROTECTORS AND IRRADIATION ON THE PLATELET MONOAMINE OXIDASE ACTIVITY

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Monoamine oxidase /MAO/ activity in blood platelets has been assumed to mirror the activity of this enzyme in the brain, and both activities have been related to the central monoaminergic activity.

The authors examined the activity of rabbit platelet MAO after incubation with various radioprotectors *in vitro*. Platelet MAO activity was measured by spectrofluorimetric method using kynuramine as substrate. It was stated that 2.5×10^{-6} M3ml AET does not cause any change but 1×10^{-5} M/ml AET resulted in complete enzyme inhibition. At the same time, WR 2721 in 6×10^{-6} M/ml and 2×10^{-5} M/ml concentrations did not effect platelet MAO activity. After 1 hr of *in vitro* 10 Gy gamma-irradiation the enzyme activity decreased by 25 %.

After the *in vitro* experiments the authors determined the *in vivo* effect of AET and WR 2721 on platelet MAO activity of rabbits. It was found that pretreatment with AET reduced the enzyme activity while WR 2721 had not such effect. The platelet MAO activity was studied after lethal dose whole-body gamma-irradiation. In these experiments the enzyme activities changed in the same direction as in the *in vitro* experiments.

THE RADIOSENSITIZING EFFECT OF ACETONE AT VARIOUS OXYGEN CONCENTRATIONS

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The radiosensitizing effect of acetone was investigated in Bacillus megaterium spores. Experiments were done in aqueous and non-aqueous liquids with four oxygen concentrations /100% N_2, 0.7% O_2, 1.4% O_2 and 2.1% O_2 in H_2/. Under anoxic conditions the extent of sensitization increases with increasing acetone concentrations up to a maximum seen at around 14 moles/litre. Increase of oxygen concentration shows a decreasing tendency in sensitization. The radiosensitizing action of acetone is due to the scavenge of hydrated electrons.

MODIFICATION OF ^{85}Sr RETENTION BY FLAVONE DERIVATIVES IN PREGNANT AND SUCKLING RATS

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It was stated earlier that the accumulation of ^{85}Sr in the bones of poultry could be inhibited by some flavone derivatives which might be radioprotective, too.

Because the $^{89-90}Sr$ isotope is one of the most dangerous fission product and its decorporation has some unsolved problems, the possibility of therapeutic application of 7-isopropoxy-isoflavone /Ipriflavon, IF./ and 2', 3.4', 5.7-pentahydroxyflavone /Morin, MO./ was investigated in rodents.

The effect of IF and MO was studied in pregnant and suckling rats on the reduction and elimination of $^{85}SrCl_2$. Pregnant rats were fed a diet containing IF or MO in a dose of 2.5 g/kg. At the end of third week of gestation, rats were exposed to $^{85}SrCl_2$. Normal rats divided into similar groups had the same treatment schedule.

It was clearly shown that the elimination of ^{85}Sr was increased by both IF and MO. The ^{85}Sr which remained in the body was only 30-50% of control value. The whole body burden of suckling rats was also decreased, and corresponded to the 50-80% of control animals.

INVESTIGATIONS ON THE DISTRIBUTION AND ELIMINATION OF ^{65}Zn IN NUDE AND THYMECTOMIZED MICE

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The distribution and elimination of $^{65}ZnCl_2$ were investigated in various organs of inbred, thymectomized and nude BALB/c mice in different intervals. The mice /in 15-15 separated groups/ were fed with normal food-preparation and water was given ad lib. The animals were injected intraperitoneally of 80 kBq 0.1 ml isotonic $^{65}ZnCl_2$ solution. The elimination of isotope from each mouse was measured daily until twice T_{eff}, using whole-body counter.

The mean half times in different animal groups were as follows: T_{eff} of control was found to be 7.9 days, of thymectomized 9.5 days, of nude mice 6.48 days. The distribution of isotope was measured in 10 various organs 24 hours after injection as well as after the first and the second T_{eff} time.

The data revealed that the thymus had an influence on the Zn-metabolism only in the brain, the bone and the skin.

Na-SUCCINATE IS A PROTECTIVE COMPOUND AGAINST ACUT ^{60}Co-GAMMA IRRADIATION VIA INHIBITION OF LIPID PEROXIDATION

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Mészáros et al. have observed, that a respiratory substrate, succinate inhibits NADPH-dependent /ADP-Fe stimulated/ lipid peroxidation of liver mitochondria. It was found, that this effect of succinat decreased after 12^{h} whole-body ^{60}Co-gamma-irradiation depending on the dose of irradiation. In the present experiments the inhibitory effect of succinate on lipid peroxidation was investigated in liver mitochondria, prepared from irradiated rats with 9.0 Gy. Lipid peroxidation was assessed by the measurement of malondialdehyde /MDA/ applying the thiobarbituric assay.

9.0 Gy ^{60}Co-gamma-irradiation results in increased MDA concentrations in liver mitochondria, during the first four days after irradiation, but the inhibitory effect of succinate was significantly diminished. The rate of lipid peroxidation remained unchanged 7 and 10 days after irradiation. These observations indicate that the inhibition of lipid peroxidation by succinate may play an important role in the defense against irradiation.

EFFECT OF IONIZING RADIATION ON THE RECOGNIZABILITY OF ALLO-ANTIGENS IN MIXED LYMPHOCYTE CULTURE

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The possibility of allogen tissue transplantation is limited by HLA difference existing among individuals, too. In model experiments using mixed lymphocyte reaction /MLR/ we studied whether the treatment of senzitizing lymphocyte with gamma radiation /0.1, 0.15, 0.20, 0.30 and 0.40 kGy/ makes any effect on the sensitizing ability. It was stated that radiation doses decrease the recognizability of allo-antigens dose-dependently. Furthermore, it was also established that the irradiation of the medium containing mixed AB serum used for lymphocyte cultures, the application of supernatant of irradiated cells to lymphocyte cultures has no influence on the stimulating effect of alloantigens.

DOSE SURVIVAL AND ENDOGENOUS SPLEEN COLONY PRODUCTION IN THE C57Bl/lOScSn MOUSE STRAIN

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Even in our days, the dose survival of experimental animals is an important question in radiation biology and biophysics. It is an essential feature that, by introducing the dose effect curves, mathematics gained a larger ground also in the "whole animal" field of biological integration /models/. Experimenting with 942 C57Bl/lOScSn mice of hygienic category II., of both sexes, aged 8 weeks: 1./ the survival in the dose interval of the hematologic radiation syndrome and 2./ the survival kinetics observed until the day of the endogenous spleen colony building /the 11.p.i. day/ were investigated. The parameters of irradiation were as follows: X-ray therapy equipment type SIEMENS Stabilipan, HVL = 0.56 mmCu, 0.256$\pm$ $\pm$0.0011 Gy/min, f + 0.936 rad/R, ambient temperature 20 ^{o}C.

According to our data: 1./ in the dose interval of 0.94-4.21 Gy, until the 11 p.i. day, there is no mortality; 2./ within the dose interval of 4.68-8.42 Gy the dose effect curves of both sexes have similar characteristic declines; 3./ the males are more sensitive, they start dying already on the 4 to 6th day /females on the 7 to 9th day/. In the case of higher doses this difference becomes indistinct. 4./ three characteristic groups of curves can be formed: I./ dose interval 4.68-5.62 Gy, II./ 6.55-7.02 Gy and III./ 7.49-8.42 Gy. In the last group the pairs of curves fall extremely close to each other. The observed differences can not be explained solely by the hematologic radiation sickness, the effect of the "whole animal" mortality mechanism should also be taken into consideration. On the dose effect curves of CR-pair BlOLP/Sn strain /V.linkage groups, H-3^{b}, A^{w}/ data will be reported later.

NUCLEAR-ANALYTICAL POSSIBILITIES OF A CYCLOTRON FOR AGRICULTURAL PURPOSES

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The first cyclotron laboratory of Hungary will start working in Debrecen in 1985. The accelerator will be a small compact cyclotron /accelerated particles:p,d, ^{3}He, ^{4}He/. Beside fundamental research, application /medical, industrial, agricultural/ projects will be running in this laboratory. The agricultural application includes radiation induced mutations, metabolism tracing by means of compounds labelled with cyclotron-isotopes, trace element analysis of agricultural samples by means of nuclear methods. The analytical methods to be applied here are: PIXE, PIGE /Particle Induced X-ray and Gamma-ray Emission/, CPAA, NAA /Charged Particle and Neutron Activation Analysis/, RBS /Rutherford Backscattering/. These methods are suitable for trace analysis in different atomic number ranges. The sensitivities of the methods are varying from ppm to ppb. An important application is the investigation of vital and toxic elements in vegetables and agricultural products used for nutrition. Absorbtion process of artificial fertilizers can be followed by means of trace analysis or by activating the fertilizer and measuring the radiation of the plants. The protection of environment possesses a special importance due to the application of dangerous chemical compounds in modern agriculture, where a considerable help can be provided by the application of the sensitive methods mentioned above.

ISOTOPE PRODUCTION FOR AGRICULTURAL PURPOSES

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At the end of 1985, after finishing the installation of the first Hungarian cyclotron in the Institute for Nuclear Research, it will be possible to produce many radioactive isotopes. They will be used in nuclear medicine for diagnostic purposes, but another possible way to use them will be in agricultural research. In the agronuclear investigations the chip reactor isotopes have the priority. However, the Hungarian reactor used for isotope production will soon be closed down for reconstruction works, and the reactor isotopes will be available only from import. So, it seems to be possible and even necessary to use cyclotron isotopes in the agricultural research, too.

Among the ultra-short-lived isotopes the ^{13}N and ^{11}C can be used for examination of ^{13}N dinitrogen fixation and for ^{11}C-methyl-l-methionin labelled investigations.

In the plant physiology investigations ^{22}Na, ^{52}Mn, ^{52}Fe, ^{56}Co and ^{65}Zn isotopes can be used for detection of different synthesis /photo-, nucleic acid-, protein-/ and decomposition /protein, starch, fat, respiration/ processes.

The incorporation of the heavy metals, which can get into the plants due to environmental pollution we can follow by using ^{197}Hg and ^{203}Pb isotopes.

APPLICATION POSSIBILITIES OF THE STABLE ISOTOPE MASS SPECTROMETER IN THE FIELD OF AGRICULTURA AND FOOD ANALYSIS

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The stable isotope ratio mass spectrometer, under development in our Nuclear Research Institute, is a four collector dual viscous inlet mass analyser with two ion sources, 180^{o} deflection geometry, 5 cm and 15 cm deflection radii, for high precision analysis of stable isotopes of H, C, N, O, S an used in the field of agriculture, food science, archeology, hydrology and geology.

Among the stable isotopes of biological interest ^{15}N is characterized by an exclusive use in agricultural studies. Gas preparation method using lithium hypobromid are under development for conversion of ammonium salts /produced by the Kjeldahl digestion/ into molecular N_2. The system will be applied for nitrogen fertilizer studies using ^{15}N labelled fertilizers.

Natural processes like photosynthesis evapotranspiration fractionate the carbon, hydrogen and oxygen isotopes in living material. Food products are synthesized by living system, therefore the stable isotope ratios could confirm the occurrence of particular biochemical pathways and consequently the origin and the labelling of a given food product.

DETERMINATION OF AMINO ACIDS IN LUPINE BY NIR TECHNIQUE

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The determination of amino acid composition is of major interest to those involved in the food and feed industry. The most important amino acids - because of their high nutritive significance - are considered to be the 10 essential amino acids.

The standard chemical determination of amino acid composition involves hydrolysis of the proteins followed by clean--up and ion-exchange chromatography. This is a fairly complicated and time-consuming process.

Recently the success achieved by near-infrared reflectance /NIR/ spectroscopy for determining protein content in a wide range of plant materials suggested research into its application for the rapid, non-destructive analysis of amino acids in lupine. This presentation introduces the application of NIR technique for the estimation of the concentrations of 17 different amino acids in lipune.

Amino acid compositions of the lupine samples were provided by Iván Koós at the Labor MIM. Results were reported in mass %. NIR spectra of the ground lupine samples were obtained on the Model 6450 Research Composition Analyzer /Pacific Scientific, USA/ at the Central Food Research Institute, Budapest, Hungary. Spectra were recorded as the log (1/R) and transposed to the 2nd derivative of the log (1/R) signal for analysis. Optimum wavelength for estimate of individual amino acids were determined by multiple linear regression analysis.

Fairly low standard errors of estimate and high coefficients of correlation were obtained for almost all amino acids. However, the results for the very important "limiting" amino acid methionine were poor. /Coefficient of variability is about 14/. This may be related to the inherent higher error of methionine analysis by ion-exchange chromatography due to

hydrolysis losses. The results indicate that the NIR technique can be used to estimate amino acid composition in lupine and perhaps in a number of other materials.

SIGNIFICANCE OF WATER BINDING OF BIOLOGICAL SYSTEMS IN THE AGRICULTURE

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The laws of water binding have been studied for long, and a lot of knowledge /considered as classic one/ helps to solve the problems of drought- and frost-resistance, as well as to work on genetics and eugenics. Basic new facts have been discovered about the structure of water during the last decades, and it was disclosed mainly by the application of the arsenal of radiospectrometry that the water content of most of the living systems is distributed among three compartments: 1./ water bound to structure 2./ the vicinal water 3./ the bulk water. The actual distribution of water among these three compartments is a characteristic property of every biological system and its modification by temperature, radiations, pH, etc. can result in profound structural changes in living systems. We shall present a review of experimental technics, the results found in the Biophysical Institute of the Medical University of Pécs, then we discuss the future perspectives.

TEMPERATURE SHOCK TO INDUCE POLYPLOIDY IN FISH

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The presence of polyploidy among fishes is frequently observed. Thus, there are possibilities - by the manipulation of chromosomes - for the induction of different polyploid forms. The viable natural or induced polyploids would bear some characters important from the practical point of view, too. Gynogenesis is a special occurrence of polyploidy. For induction of polyploidy, temperature shock is commonly used. In the Fish Culture Research Institute /Szarvas, Hungary/ methods to induce gynogenesis, as well as for production of viable triploids having good growth rate, was elaborated and applied on European catfish. The method of gynogenesis consisted of radiation of the sperm with γ-rays of Co^{60} /100.000 rad/. This radiation destroys the haploid genetic material, then the fertilization was carried out by using this sperm. Five minutes after the fertilization the fertilized eggs were cooled down to +4 ^{o}C and kept at this temperature for 30 minutes. The heat shock was not successful to induce polyploidy. The method of the production of triploid European catfish was similar to that of the gynogenesis but the fertilization was carried out by unradiated, fresh sperm.
In both cases the polyploidization was induced by the retention of the second polar body. Triploidy has been proved by karyological and erythrocyte size analyses.

ESTIMATING LOW TEMPERATURE TOLERANCE OF PLANTS FROM CHANGES IN CHLOROPHYLL FLUORESCENCE

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The chilling-sensitivity of plants has been correlated to the physical state of cellular membranes. The phase transition temperature may be useful as a rapid screening method for establishing the tolerance to low temperature. The induced prompt and delayed fluorescence emitted by the chlorophyll in vivo has been used extensively for studies of photosynthesis. Also in our case the fluorescence of chlorophylls in the chloroplast thylakoid membrane seemed to be a suitable tool for investigating the response of plants to low temperature stress. The chlorophylls play the role of naturally occurring intrinsic fluorescent probes. It was shown that both fluorescence induction kinetics and the intensity of delayed luminescence of intact leaves provide a rapid and sensitive method for detecting the low temperature and chilling tolerance of plants.

RELATIONSHIP BETWEEN ELECTRIC CONDUCTIVITY VALUES AND SEED VIGOR IN PEAS /PISUM SATIVUM L./

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In a series of experiments seed samples of five pea varieties bred at the Debrecen Agricultural University were studied for correlations between electric conductivity of the soaking water and germination. In the case of four varieties seed samples from three cropyears, in the remaining one variety samples from nine cropyears were investigated for correlations between electric conductivity and germination percentage, seedlings raw weight and length.

In addition a smooth and a wrinkled seed variety were subjected to artificial ageing.

The "electric seed vigor" test value expressed in uS/g provides reliable information on seed viability only in the case of homogeneous seed lots. Environmental conditions during harvest and storage, state of health and especially genetic constitution may considerably modify the measured conductivity value. Marrowfat peas due to their looser texture show a higher conductivity value than the smooth-coat round seeded peas. With the advance of natural as well as artificial ageing the conductivity value increases in close correlation with a decrease in germination percentage and average seedling weight. Within the different varieties seed specimens show considerable variability both in conductivity and seedling size. Significant correlation between conductivity of seed specimens and seedling weight and length, respectively, was not in each case demonstrable.

Our investigations showed that the electric conductivity may supply good information on the state of the tissues. This, however, should be completed with other variability tests to obtain preliminary information on the field behaviour of the seed samples.

To select and classify the seed specimens quickly, according to their individual relative electric conductivity, last year a new measuring apparatus was developed.

THE Ca-ACTIVATED PHOSPHORYLATION SYSTEM IN WHEAT CELL

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The protein-bound phosphate turnover is catalyzed by protein kinases /pk/ and protein phosphatases /pf/. The phosphorylation of an enzyme often causes a dramatic change in K_m for a substrate. Phosphorylation-dephosphorylation should therefore be regarded as a mechanism for switching an enzyme between two forms. Among the pk-s the Ca-activated kinase is responsible for the phosphorylation of plant endogenous proteins in membranes and in soluble fractions whose identity and function are not yet known.

Our aim was to purify the Ca-activated pk and to find its targets which are supposed to be involved in cellular metabolism.

Tissue cultured wheat cells were fractionated by centrifugation to obtain membrane and soluble proteins. This latter fraction was further purified on DEAE cellulose column. The Ca-activated pk was present both in membrane and soluble preparations. The Ca in 100 μM concentration maximally stimulated pk activity in the presence of 5 mM Mg. EGTA inhibited Ca-dependent activity showing Ca to be essential for the pk. EDTA added in 10 mM concentration after 15 min preincubation decreased the $^{32}P_i$ incorporated into proteins via blocking the phosphorylation but not the dephosphorylation. The status of the different phosphoproteins was detected by 2-D PAGE and autoradiography.

THE ELECTROSTATIC TREATMENT OF POTATO SEEDS

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We have examined the effect of electrostatic treatment on the seed tuber in order to know the extent of its influence on the accumulation of potato tubers, the growth of stems, the number of seeds and the mass, as well, as on the internal content of the crop. We treated the potatoes with an electric field intensity of $E = 1.67 \cdot 10^5 \frac{V}{m}$ or $1.82 \cdot 10^5 \frac{V}{m}$, 1-2 days before sowing.

We did our experiments in the open air, four times repeated, in blocks with 2-4 species disposed randomly. We evaluated the results with mathematical methods.

Results: In the cases of the Desire and Somogyi roll seeds the 30 min and the 60 min treatments, respectively, were the most effective for the seed accumulation intensities in the first year. In the first period of each treatment, in the general longitudinal increase of the potato stems, it was possible to show the stimulating effect. In the case of the Desire the number of leaves on the stems increased /0.6-1 pieces/stem/. The average yield of the Desire was 3.9-8.5 % better than the control in the first year. The results of the first year were justified by the experiments of the second and the third year. According to the species, the increase of the yield changed between 2-33 %, which ment some 0.4-6.8 t surplus for a hectare. The dry material content of the seeds decreased with 1.1-2.1 %, the gross protein referring to dry material increased 0.31-0.58 %, as compared to the controls.
Consequences: By the results of measurements it is observable that the electrostatic treatment influenced the growth of vegegative parts, the number of seeds on parcels, the mass of the yield, and the content of gross protein positively.

SOME NEW ASPECTS OF THE CORRELATION OF BIOCHEMICAL CONTENT: CHARGE RELATION IN THE BIOLOGY OF REPRODUCTION /SPERMATOZOA, EGGS AND EMBRYOS/

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An electronmicroscopical /TEM, SEM/ semi-quantitative label technique for measuring the dispersion of surface charges on molecular level /1/ has already been developed by the author.

Actually, the surface charge was studied and its changes using fresh, deep frozen, and acrosome reacted spermatozoa of man, mouse, bull, boar, wild boar, rabbit deer, roe-deer, rooster, goose, turkey, fishes, respectively, a not alse eggs and embryos. The free anionic sites, i.e. the dissociated terminal carboxyl groups of sialic acid, are measurable on the whole surface of the plasma membranes of the objects studied. The density of the coulombic forces is, however, very low on the heads of fish spermatozoa, a high density can be shown at the middle-, principal-, and especially at the end-piece of the spermatozoa of all species and at the perforatorium in the case of bull and deer. On the surface of eggs and embryos there is a random distribution of the negative charges.

Acrosome reacted spermatozoa cells lost plasma- and outer acrosome-membranes /Galea capitis/. They show, at the same time, negative and positive charges; at the acrosome region, however, the density of the negative charge holders is higher.

The surface of tissues of the epididymic as compared with that of the uterus also proved to be negative.

The interpretation of the findings show the increasing role of the study of different charges in biology.

/1/ Veres, I.: Electronoptical Measurement of Surface Charges. In: Hayat: Principles and Techniques of Electron Microscopy Vol. 9. Chapter 8. p. 262-299. Van Nostrand Reinhold Comp. New York, etc. 1978.

INCREASING THE EFFICIENCY OF SEED DRESSING AGENTS USING ULTRASOUND IN THE CASE OF SUNFLOWERS INFECTED WITH PERONOSPORA

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The effect of ultrasound to increase permeability and diffusion was applied for the introduction of 15 kinds of dressings as well as plant protecting agents into the achenium and seed of the sunflower variety GK-70. Then, in their early leaved age, in our first investigation, the spotted germ plants were infected with peronospora.

Our results indicate that, using ultrasound to introduce the dressing agents at a concentration of 0.1-0.2 % containing metalaxil Cu, Zn and Benomyl /Ridomil 50 WP, Ridomil Plus, Cuprosan SD, Cursate CZ, Fundasol/, appear to be able to ensure quite satisfactory protection for the plants in field experiments, despite the serious infection. We have similar results using ultrasonic dressing of sunflower infected artificially or naturally by Plasmopara halstedii and Sclerotinia sclerotiorum.

The optimum dose of ultrasound determined on the basis of germinativ features, and applied at a frequency of 21.5 Kc/s and duration 5 minutes exerted a similarly favourable influence on the vigour value and germination of the seed grain and the development of seedlings.

The applied US-dressing may help the plant protection technology in increasing the immunity of plants and is also suitable for comparison tests of efficiency of dressing agents.

SEVERAL RESULTS OF USING ULTRASONIC SEED TREATMENTS IN AGRICULTURAL RESEARCH AND PRACTICE

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In the last decades many publications have dealt with the possibilities of stimulating plant seeds, or growing plants in their germination, development and, eventually, in their yield, by treatments with physical methods e.g. ultrasonic irradiation by 800-1000 Kc/s generators originally used for medical treatments. A new possibility was the development of higher power 20-40 Kc/s industrial US-generators of larger irradiation volume for seeds.

Our experiments for stimulation several seed corn species were done by KLN "Ultraschall Laborgerät" with a constant frequency of 21.5 Kc/s and in a ultrasonic irradiation tank of 4 liter volume. The increased permeability of seeds under sonication described in literature was used for the input of several microelements and in further experiments seed dressing chemicals, too.

As an effect of irradiation in microelement solution at optimal duration and intensity of sonication a significant increase was found in germination, growing, and yield e.g. maize /4-15 %/ and radish /10-30 %/, etc. There are good results in breaking dormancy of several variety of hard seeds by sonication. /Red and white clover, alfaalfa, etc. belonging to leguminous fodder crops./

Ultrasonic seed dressing using solution of most convenient chemicals resulted in better protection and development of seedlings and plants for example in sunflower, bean, and rice infected by mildew, fusarium, nematode worm etc.

These techniques could already be used especially for dressing valuable seeds of smaller quantities, for stimulating horticultural seeds and for facilitating germination by softening hard shells in the practice.

EFFECT OF ULTRASONIC SEED TREATMENTS ON SOME PAPILIONACEAE VARIETIES WITH A HIGH HARD SEED CONTENT

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In seed lots of leguminous fodder crops the occurrance of hardcoated seeds is frequent.

For reducing seed hardness several methods have been worked out, but the practical realization of most of them is difficult and their results are not always satisfactory.

Comparative analysis of traditional seed treatment processes and ultrasonic treatments have been carried out for the determination of methods that can with good results be applied at the seeds of varieties of leguminous fodder crops for reducing seed hardness.

In the authors' experiment it was investigated by what parameters of ultrasonic treatment can the germinating characteristics be most favourably modified, the seed hardness be resolved, and how - in greenhouse conditions - its effect appears in plant emergence.

In small plot experiment we investigated the effect of ultrasonic seed treatments on growth vigour, flower and fruit formation in red clover.

In the comparative experiment the ultrasonic treatment proved to be the most favourable, it is simple and fast to implement and there is no injury of germs.

With the resolving of seed hardness the germinating power and the germination speed increased.

Emergence rate of lots treated with ultrasound increased with the improvement of germination, the germs grown from treated lots were more vigorous.

Appearance of foliage leaves, the onset of flowering, inflorescence number by plant, the plant number's closeness at harvest and the seed yield per plant were more favourable in the stand developed from treated seeds, than in the untreated ones.

CORRELATION BETWEEN PHYSICAL AND BIOLOGICAL PROPERTIES OF MAIZE /ZEA MAYS L:/ SEEDS

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Seed physical properties play an important role in seed processing. There is a worldwide scientific research for new physical markers giving more information about inside structure of seeds. Such markers would help to improve the efficiency of seed processing and determination of biological value.

In recent studies some physical characteristics of maize seeds were tested and compared to viability and vigour values.

Physical tests: evaluation of density, electrical conductivity, vibration /audible range/.

Biological tests: evaluation of germination, cold test, complex stressing method /Szirtes-Barla/.

The results suggest that the seed density, due to the proportion of components of materials and structure, is in close correlation with viability and vigour.

The electric conductivity /measured by ASA 610/ gives information about membrane integrity. Accuracy of measurement depends on the size and weight of seeds tested. Bigger seeds diffuse more ions affecting electric conductivity.

Vibration of audible range was analyzed by AGROPROCESSOR VI. instrument based on quick-Fourier transformation. Analysis of vibration gives a possibility for more rapid component quality and seed viability tests.

INTERNAL GAS COMPOSITION OF THE WHEAT STALK AND ITS POSSIBLE CONNECTION WITH YIELD AND ADAPTABILITY

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Using a built-in and insulated gas sampling capillary covered with a silicone membrane and connected to a quadrupole mass spectrometer, internal gas composition of the wheat stalk and its change was continuously measured in vivo. As compared to the ambient air, the wheat stalk contains significantly more CO_2 and H_2O /water vapour/ and somewhat less O_2 and N_2. The internal gas composition changes with the age of the experimental plant. Maximum CO_2-values of 1 to 2 vol. % are reached about one week after anthesis at a value of 0.05 vol. % in the lab atmosphere. Various physical effects exerting the whole plant or its parts /leaves, roots/ also modify the stalk atmosphere. Under illumination /3000 lux/, CO_2-level in the hollow stalk decreases, while the O_2-level increases after a short lag-phase. Subsequent darkness results in an opposite change. By cooling some leaves or moistening them with a herbicide /i.e. Gramoxone/ solution, specific, stepwise reactions can be induced with a characteristic increase of the CO_2:O_2 ratio in the end phase. In water-stressed plants, CO_2-concentration of the stalk cavity decreases. Consequently, internal gas atmosphere of the wheat stalk is not an engulfed part of the outer atmosphere acting statically against lodging, but an environment-sensitive, dynamic system, both depending on and influencing photosynthesis and respiration.

STUDY OF TRANSPORT PROCESSES IN SOILS AND PLANTS BY MICRORADIOGRAPHIC AND RADIOABSORPTION METHODS

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The concentration profiles of lead and boron is carrot root and potato tuber were determined at various diffusion times by microradiographic method. The order of magnitude of the diffusion coefficient of the lead ions and the borate- or tetraborate ions was $10^{-3} m^2 s^{-1}$ and $10^{-11} m^2 s^{-1}$, respectively. The transport process of nutrients, leaf-manures and plant--protecting agents in plants was investigated by radioabsorption method. The effect of complex-forming agents on the absorption of nutrients and leaf-manure compositions was also examined.

The influence of the humidity, perviousness, pH and temperature of the soils, as well as the action of soil-additives and complex-forming agents on the effective diffusion coefficients of nutritives was studied by means of the radioabsorption technics. In soils, the effective diffusion coefficient of the nutrients was found to change in the region of $10^{-16} m^2 s^{-1}$ to $10^{-10} m^2 s^{-1}$.

The numerical data of the above measurements give valuable information about the transport processes in plants and soils, allowing the control and influence therefore, and thus contributing to the utilization of the results in agricultural practice.

THE APPLICATION OF THE PROTON INDUCED X-RAY EMISSION METHOD IN ANALYSING HORTICULTURAL SAMPLES

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As a consequency of the introduction of new technologies for producing selected new species of plants, nutrient economy became an important central question in horticulture. New species are genetically more particular than the old ones and new environmental conditions caused by new technologies overburden plants. Therefore, good results can only be expected if plants are harmonically provided by all essential elements. The detailed knowledge of the husbandry of microelements may help us in fulfilling this condition.

Proton induced X-ray emission method represents an effective tool in the simultaneous analysis of all the elements heavier than magnesium with sensitivity nearly identical in ppm level for all the elements.

As part of the research work performed in the Institute of Vegetable Growing, PIXE analyses were performed on leave samples of water melon using proton beams of the 5 MeV Van de Graaff accelerator at the Institute of Nuclear Research. Fourteen elements /Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Ni, Cu, Zn, Br, Sr/ were analysed in twenty six samples prepared from dried leave samples. The preliminary results obtained in these measurements fully prove the applicability of the method in the present case. Further experiments are being planned to clear up details of the husbandry of microelements in water melone.

EFFECTS OF THIOCARBAMATE HERBICIDES ON THE MEMBRANE AND SURFACE LIPID COMPOSITION AND TRANSPIRATION OF MAIZE /ZEA MAYS L./ SEEDLINGS

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The quantity and quality of epicuticular and membrane lipid classes and components /Barta, I.Cs. and Kőmives, T., J. Chromatogr., 1984, 287, 438/, as well as transpiration rates of leaves of maize seedlings treated with the thiocarbamate herbicides EPTC /S-ethyl-N,N-dipropyl thiocarbamate/ and butylate /S-ethyl-N,N-diisobutyl thiocarbamate/ were determined.

As a result of treatments with 10 μM concentrations of EPTC and butylate, transpiration rates of 14-day old plants were increased by 201 and 138 %, while shoot heights were only decreased by 21 and 9 %, respectively. These herbicides were also found to be effective inhibitors of fatty acid elongation and desaturation /Barta, I.Cs., et al., Acta Biochim. Biophys. Acad. Sci. Hung., 1984, 19, 131/. Time course and dose-response studies indicated that among the membrane lipids linoleic acid content was most sensitive to thiocarbamate herbicides, while among the epicuticular wax components the levels of C_{31} hydrocarbons were most strongly affected.

It is suggested that increased transpiration rate by thiocarbamates is due to alterations in the membrane and surface lipid composition.

ABSORPTION AND TRANSLOCATION OF HERBICIDE ANTIDOTES IN CORN PLANTS /ZEA MAYS L./

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The fate of bioactive materials in living organisms is determined by biophysical /absorption, translocation/ and biochemical /bioactivation, bioinactivation/ factors. To be effective the xenobiotics must reach the sensitive site/s/ of action in its active form, at a concentration sufficient to cause severe primary and/or secondary effects which result in injury or disruption of normal life processes. Barriers such as absorption and translocation may influence the active /critic/ internal concentration of the bioactive species. The importance of these phenomena in the biological activity of herbicide antidotes has been investigated. Chemical antidotes are compounds eliminating the injurious effects on cultivated plants by herbicides without decreasing the successfulness of weed control.

Germinated grains of maize were grown in Hoagland solution for one week then transferred into similar nutrient solution but containing 5×10^{-4} M of the antidote examined. Active ingredient content of plants /root parts and sprout ones separately/ and nutrient solution was determined by GLC after extraction and clean-up of plant-extracts.

Quantity of MG-191 experimental antidote in the buds changed parabolically during the first five days. Maximal volume of the active ingredient both in the shoots and in the roots was found on the third day. MG-191 content in the roots changed more quickly than in the offsets. Although the absorbed volume of AD-67 /N-dichloroacetyl-1-oxa-4-aza-spiro-4,5-decane/ and R-25788 /N,N-diallyl-2,2-dichloroacetamide/ antidotes into the whole plants was about ten times more than that of MG-191, the latter produced the highest efficiency. Translocation of all the agents studied was rapid. Thus, their transport up to the sensitive site/s/ of action is not hindered.

EFFECTS OF PHYSICHOCHEMICAL PARAMETERS ON THE ABSORPTION AND TRANSLOCATION OF HERBICIDES

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Using ^{14}C-labelled acetochlor /2-chloro-N-[2-ethyl-6--methyl-phenyl]-N-ethoxymethyl acetamide/ and EPTC /S-ethyl--N,N-dipropyl thiocarbamate/ the uptake and translocation of these herbicides /I. Jablonkai, F. Dutka, J. Radioanal. Nucl. Chem., Letters 94, 45 /1985// in maize /Zea mays L., Pioneer 3950 hybrid/ and mustard /Sinapis alba L./ plants, as well as their lipophilicity and water solubility were studied.

Uptake of acetochlor /maize: 0.45 μmole/g fresh weight; mustard: 1.16 umoles/g fresh weight/ was found to be more rapid than that of EPTC /maize: 0.26 umole/g fresh weight; mustard: 0.13 μmole/g fresh weight/ after 3 h exposure to 0.4 mM concentration of the herbicides in nutrient solution. Translocation of acetochlor from roots to shoots /maize: 14%; mustard: 61%/, however, was less ready than that of EPTC /maize: 43%; mustard: 74%/.

Lipophilicity /octanol-water partition coefficient: 26.1/ and water solubility /1.98 mmoles/l/ of EPTC are higher than those for acetochlor /15.9 and 0.83 mmole/l, respectively/.

In accordance with literature data /I.J. Graham-Bryce, in Pesticide Synthesis Through Rational Approaches, Eds. P.S. Maggee et al. Am. Chem. Soc., ACS Symposium Ser. No. 255, 1984. p. 185/, our results show no simple relationship between physicochemical properties and uptake, and indicate that translocation is determined by water solubility.

THE EFFECT OF DIFFERENT DOLOMITE DOSES ON THE WILTING OF SUNFLOWER

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In the experiment two months old sunflower plants /Helianthus annuss cv. Mariana/ were used. While the control group was developed in a Ca and Mg deficient sand with a pH value of 4.8 the soil of the two other groups was amended with a dose of dolomite equivalent to 300 kg per hectare and 600 kg per hectare, respectively.

The plants were cut between the stem and root and left to wilt at a laboratory temperature of 23 ^{o}C in a uniformly lit environment of approximately 100 Lux. The assimilating surfaces of the different groups showed no statistically significant differences.

The sequence of wilting was the following:

treatment /dolomite/	wilting time /hours/
1. 300 kg/ha	20 - 24.5
2. control	23 - 28.75
3. 600 kg/ha	28.5 - 33.5

The explanation of the obtained results can be as follows: In the case of the smaller dolomite dose the absorbed bivalent cations overshadow the negatively charged groups of the biocolloids and, as a consequence, their waterbinding ability decreases. That is the reason for the faster wilting of this group, as compared with the control. In the case of the greater dose, however, the Ca^{2+} and Mg^{2+} ions act as bridge creating agents and form microcapillary network with the pectine macromolecules of the cell wall interwall formation /interlamina/ and, in consequence of this, the water holding ability increases.

CRYOPRESERVATION OF EUROPEAN CATFISH /SILURUS GLANIS L./ SPERM

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During the last few years from the carnivorous European catfish wild population, domesticated strains were developed, which can be raised on pellet and artificially propagated, therefore are suitable for semi- or superintensive fish production. The application of these technologies required the elaboration of the cryopreservation of European catfish sperm. As extender the Alsever /AL/ solution /Frankel 1970/ proved to be the most suitable from the generally used solutions. As cryoprotective agent dimenthylsulfoxide /DMSO/ or ethylenglicol /EG/ were used. Optimum ratios of extender and cryoprotective were AL 15 % DMSO and AL 15 % EG, respectively. The dilution ratio for sperm ranged from 1:1 to 1:5. It was determined that the crucial point of cryopreservation is the freezing speed. The freezing must be carried out continuously. Freezing rate must be 10 ^{o}C/min before reaching -80 ^{o}C, then 20 ^{o}C/min to -150 ^{o}C. After this the sperm is put and stored in liquid nitrogen. In case of quicker freezing rate the semen was precipitated and became useless for fertilization.

Using the cryopreserved semen the same fertilization rates were obtained as with the control untreated fresh sperm.

A SUMMARY REVIEW ON THE SPATIAL DISTRIBUTION OF THE NEGATIVE AIR IONS IN THE POULTRY HOUSES

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In the first place it must be remarked that when forming environmental parameters in poultry-farming, different requirements exist from these of human environmental parameters.

In 1975 the University for Agricultural Sciences - Department for Zoobiology and Animal Physiology - recommended and prescribed 400-700 ions/cm^3, but in 1984 5000 ions/cm^3 were recommended. They also prescribed the type of the ion-covering.

By forming the inner ion-climate in poultry houses the following four ways can be chosen: 1.: to disperse - in every direction - the negative air ions by means of ventilation shaft of ventilator, 2.: to disperse negative air ions with natural atmospheric motion, 3.: to construct models of punctiform ion-source, 4.: to increase the internal ion distribution by the effect of diffusion and convection. The most effective procedures can be enlisted in item 4 which must be designed and computed by computer. Thereafter fitting places of the ionizers and ventilators should be constructed.

INFLUENCE OF ARTIFICIALLY INDUCED UNIPOLAR AIR-IONS ON MASS--GROWTH AND FEED-CONVERSION OF BROILER-CHICKENS

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The natural concentration of air-ions changes around the animals living in climatized and insulated coops: the number of negative O_2-ions decreases and the number of positive CO_2-ions increases. A change in their proportion would influence the metabolism in animals in an unfavourable way.

In my experiments it was my aim to find the kind of negative ion-concentration in the air which is optimal for the mass-growth and feed-conversion of broiler-chickens. So, experimental groups of 10 HYBRO-broiler pullets and 10 cocks were founed. The concentration of negative-ions in the air was 100 in the case of 3 groups, in another 3 groups it was 5000 and in yet another 3 groups it was 25000 negative ions/cm^3 in the air. At the end of the 42nd day of the experiment body--mass and food-consumption were measured.

The results indicate that the body-mass of control /100 negative ions/cm^3/ pullets and cocks was 1331 g and 1625 g, respectively, and their joint specific-body-growth was 504.3 g mass/kg of food, while these same indicators were by 3.6 %, 1.75 % and 5.69 % higher in the environment with 5000 negative ions/cm^3 and in the case of 25000 negative ions/cm^3 the first two indicators were by 0.23 %, 0.31 % worse and the third one was by 4.26 % better than in the control group. The differences between the control group and the one kept in an environment with 5000 negative ions/cm^3 are significant at P = 5 %. To sum up this way, it is advantageous to keep up a homogenous concentration of 5000 negative ions/cm^3 of air throughout the breeding of broiler-chickens.

CONTROL OF MINERAL METABOLISM OF POULTRY WITH SOME BIOPHYSICAL PARAMETERS OF BIOMINERALIZATION

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Minerals - among them Ca and P - have significant structural and functional roles in animal organs. Among the farm domestic animals poultry /Gallus domesticus/ is outstanding for its vigorous metabolism. Most investigation procedures are based on data from chemical analyses of blood. But these gave information only when marked major changes have occurred, since their blood levels, undergo hormonal regulations. The dynamics of mineral metabolism depends mainly on the mineralization of bones so the determination of physical parameters facilitates their estimation.

The authors determined the pressure and flexibility strength of some bones /tibia and femur/ by mechanical method in animal populations which had been supplied with different levels of minerals and also in a population of different level of production.

The values were compared to the data received by chemical analyses of bones and blood. It was found that a/ the results of the chemical analyses of bones can be used with greater safety than the results of the blood analyses. b/ The bones of different regional anatomical positions in the living organism are affected by different diverse actions which makes assessments uncertain. The egg producting structure is determinant in the poultry. At a given level of mineral material supply the pressure strength of tibia is greater /22.5 MPa/ than that of the femur /12.8 MPa/, while the flexibility strength of the femur is strikingly high /45 MPa/.

PHYSICAL METHODS FOR THE SEPARATION OF X- AND Y-CHROMOSOME BEARING SPERM

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Differences in physical, biochemical and immunological properties between X- and Y-chromosome bearing spermatozoa have been postulated by several investigations. One of the proven differences between the X- and Y-chromosome bearing male germ cells is the difference contributed by the sex chromosome. Difference in DNA quantities between the two classes of sperm cells is 2.5-4.5 % in most mammalian species. Staining sperm DNA with a fluorochrome, flow cytometry offers a method that is capable of observing the above mentioned high differences in DNA content of X- and Y-bearing sperm. Combination of flow cytometric DNA content analysis of sperm populations with cell sorting provides a possible method of separating the X- and Y-chromosome bearing sperm subpopulations. Another promising method for sex orientation of mammalian sperm is based on the different swimming behaviour of X- and Y-sperms in a laminar flow cell. Personal experiences with the above techniques and preliminary experiments are discussed in the paper.

MEASUREMENT OF HEAT LOSS TO FLOORINGS IN PRACTICE

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In zoohygienical environmental diagnostics it is important to state the heat-absorption of air and floor or litter. A thermoelemental heat-convection-meter in the air indicates stationary heat-flux. On the floor it first indicates a maximum after 15-20 s, then a gradual decrease and, finally, a practically stationary state follows. The less the heat-absorption factor of the floor or the litter /b; $kJ/m^2.h^{1/2}$/ is the earlier and lower the stationary heat-flux takes place.

The diagram of heat-flux is similar with the preheated-box-convection meter, too. The heat source of this apparatus is a box, instead of an animal. The temperature of the box can be kept stable by an electric heater.

In practice, only simple instruments are usable. Such is the hot-water box, which indicates a part of the initial, period in the instationary heat-flux on the basis of temperature decrease.

Such a value can be measured by the Hill's katathermometer, too. It should be put into the litter, so that its fluid-level should be in the vicinity of the starting temperature.

Because of the relatively long space of measurement some litterstocks may be compared only in standard air-condition, e.g. in a climatized room. For this reason the stationary heat-values on the floors or in the oitterstocks are to be compared with the simultaneous heat-values in the air.

In this way, at an air temperature of 19.5 ^{o}C the instruments give a heat quotient of 0.84 between strow and air, 2.32 between sand and air, 3.73 between concrete and air.

SOME PHYSICAL PROPERTIES OF FATTY POWDERED MILK

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Extended application of physical methods in food industry, particularly by the applicability of ionizing radiation for the preservation of foods, have come into prominence. The large sensitivity of fats and fat-containing foods to the influence of radiation treatment is well known. Our purpose was to investigate the temperature dependence of the AC electric conductivity and dielectric constant of fatty powdered milk, and the free radicals induced by radiation by electron paramagnetic resonance /EPR/ method in the function of radiation dosage. The mutagenic effect arising from the influence of radiation was studied by Drosophila mosaic test. At the same time, the chemical and microbiological characteristics were also investigated.

The sample were irradiated by ^{60}Co gamma radiation source to different extents /0.1-2 kGy/. The physical properties were measured by a TESLA type BM 484 semiautomatic precision bridge, in pressed layers equipped with silver electrode in the temperature range of 200-325 K. EPR measurements were carried out on a JES-PE-IX /Jeol, Japan/ X band spectrophotometer.

We have obtained the following essential results:

- Temperature dependence of electric conductivity /T/ shows similar character to organic semiconductors
- Dosage dependence of activation energies calculated from /T/, and free radicals given by EPR spectrum show similar saturation curves /at 1 kGy/.
- Free radicals disappeared from the dissolved and again solid powdered milk - this was supported by the decrease in the activation energy.
- Rise in the frequency of mutation was not observed.

INVESTIGATION OF WATER BINDING IN PLANT SECTIONS

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In our present experiments we have examined the vapour pressure of the remaining water of the gradually dried plant sections /potato/. We cut sections of 20x10x1 mm^3 from the potato by the aid of a metal pattern. We dried the segments gradually step by step and at each step we measured the vapour pressure values of the segments at different temperatures, and determined the temperature coefficient of the vapour pressure. Using the Clausius-Clapeyron equation the evaporation heat of remaining water in the plant section can be calculated. We found that the evaporation heat increased significantly together with the decreasing water content of the plant sections. We obtained similar results in the case of muscles. In our opinion these results also support the gradually increasing boundedness of remaining biological water.

STIMULATION TREATMENTS ON LARGE-SEED LEGUMINOUS PLANTS. THE EFFECT OF ELECTRIC FIELD ON THE GERMINATION

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The effect of treatments of electrostatic field with different intensity and length of time on the seeds was examined in a series of experiments with one variety of four leguminous plants /Lupinus albus, Lupinus luteus, Pisum sativum, Phaseolus vulgaris/ each.

The aim of the experiments was to study the effect of the applied treatments on the germination and the development of seedlings, further, to find out the duration of stimulation effect after the treatment.

The treatments were carried out by equipments constructed by the Department of Physics of Agricultural University Debrecen, with the following field intensities: 2 kV/cm, 1 hour; 2 kV/cm, 2 hour; 6 kV/cm, 1 hour.

4 x 25 seeds were germinated in filter paper in incubator at 20-22 ^{o}C. The evaluation was carried out on the 7th day. The following data were studied: germination capacity, and the length of the shoots and radicles,as well as the diameter of the hypocotil 1 cm under the cotyledon of each seedling.

The first germination was carried out immediately after the treatment, then it was repeated on the 14th and 28th day, and in each 4th week for 7 montsh, and one year after the treatment.

The data were evaluated by the usual methods of the biometrics. On the basis of the variation analysis the treatments by the examined species and parameters had positive effects. The optimal dose varied according to the species.

The stimulation effect alterned between 3 and 30 %, and with certain fluctuation could be revealed even one year after the treatment.

REGULATION OF ION TRANSPORT IN WHEAT

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Transport processes play a fundamental role in growth and development of plants. In general, net accumulation of ions during the life cycle, as well as the changes in average ion concentrations in plant tissues are well documented. The dynamic properties and the regulation mechanism of transport processes, however, are less understood.
In this contribution several regulatory mechanisms will be dealt with as follows:

1. Regulation on cellular and membrane levels
   - feed back control and hormonal effects;
2. Regulation in tissues and organs
   - source and sink relationships;
   - long distance transport.
3. Changes in transport processes during the life cycle.
4. The role of environmental factors.

These possibilities will be illustrated with examples given from K^+ transport in winter wheat.

THE MECHANISM OF THE INTERACTION BETWEEN GRAMICIDIN AND RED BLOOD CELL MEMBRANE

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Gramicidin increases the cation permeability of red blood cell membrane. The exchange diffusion measured in the presence of gramicidin can be described by a two exponential function. Our model /the explanation of these kinetic results/ describes the penetration of the antibiotic into the membrane as a cooperative process. The model was proved experimentally: the number of antibiotic containing erythrocytes was estimated, this value was plotted against the gramicidin concentration and an S like curve was obtained. On the basis of ionselectivity measurements it was proved that the gramicidin molecules penetrating into the membrane created ionconducting channels in the membrane.

INVESTIGATION OF HERBICIDE-ANTIDOTE ANTAGONISM ON PLANT CELL MEMBRANE PERMEABILITY

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The antidote-reversible effects of thiocarbamate and chloroacetanilide herbicides on betacyanin efflux from aged beetroot /Beta vulgaris L. cvar. rubra/ disks /Wilkinson, R.E. and Smith, A.E., Weed Sci., 1976, 24, 235/ were studied as simple models of herbicide-antidote antagonism at the plant cell membrane.

Dose-response and time-course studies revealed that, with lag-periodes of 5-10 min, EPTC /S-ethyl-N,N-dipropylthiocarbamate/ and acetochlor /2-chloro-N-ethoxymethyl-N-[2-ethyl-6--methylphenyl]acetamide/ increase betacyanin efflux at concentrations higher than 1.0 and 0.5 mM, respectively.

Experiments with compounds belonging to a new class of herbicide antidotes /Széll, E., et al., Cereal Res. Commun., 1985, 13, 55/, as well as with commercial products, showed that antidotes may protect beetroot cell membranes against thiocarbamate and chloroacetanilide herbicide damage: less pronounced efflux of betacyanin was observed when herbicides /1.5 mM/ and antidotes /0.15 mM/ were simultaneously applied. Effectiveness of the antidotes to counteract herbicide damage depended on the chemical structure and the concentration of the antidote. A positive correlation was established between the ability of the antidotes to alleviate herbicide injury to maize /Zea mays L./ seedlings and to beetroot cell membrane.

The results indicate that one of the multiple sites os antidote action /Kőmives, T. and Dutka F., Cereal Res. Commun., 1980, 8, 627/ is membrane-related.

CHANGES OF ELECTRIC POTENTIAL AND ION CONCENTRATION DUE TO SEPARATED CHARGES IN CLOSED BIOLOGICAL MEMBRANES

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In the energy transducing membranes the conversion of energy starts with a vectorial charge separation giving rise to a transmembrane electric field. The energization is completed by proton translocation across the membrane.

We have calculated the electric potential of separated charges and the variation of ion concentrations /and pH/ across and along the membrane in a model where the dielectric membrane forms a sphere separating two compartments of electrolyte. The changing configuration of the electric field in the membrane when the charge localized at the donor side of PS1 enters the inner aqueous phase is a possible explanation for the slow rising component /phase b/ of the electrochromic absorbance change of photosynthetic pigments.

The creation of a dipole may cause depletion of cations and accumulation of anions in the electrolyte on the side of the positive charge, and the opposite effect on the other side. This provides different operating conditions for ion pumps depending on their lateral location. Hence the lateral variation of the electric potential and the consequent local variation of pH and ion concentrations alonf the membrane surface may regulate the functioning of ion pumps.

THE CHARGE TRANSPORT BY Na - K ATPase IN MODEL MEMBRANES

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Membrane fragments containing Na - K ATPase were isolated from pig kidney. They were incorporated into model membranes: the membrane fragments with the Na - K ATPase attach to the millipore filter impregnated with phosphatidylcholine in an oriented way. The electric properties of the membranes were measured with two electrodes immersed into the electrolytes on the two sides of the membrane. On addition of ATP the membrane exhibits electric activity, a current due to the charge transfer can be measured. The electric parameters of the signals were determined.

LATERAL DIFFUSION MEASUREMENTS IN MODEL MEMBRANES

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In addition to the conventional molecular dynamical and short range order measurements, spin labelling electron spin resonance spectroscopy can also be applied for determination of lateral diffusion coefficient, provided the label/lipid ratio is higher than 1:100 mole/mole. The method is based on the analysis of linebroadening brought about by dipolar interaction and spin exchange.

Here we report our first results in conjuction with computer-assisted lineshape analyses. The main conclusions can be summarized as follows: /1/ The previously published algorithm /Sackmann and Treuble, 1971/ has been generalized to include motionally averaged anisotropic lineshapes. /2/ It is obvious from these computer analyses that over a wide range of temperatures above the phase transition both dipolar interaction and spin exchange make significant contributions to the linebroadening. /3/ Using different positional isomers of spin labelled stearic acids a motional profile was found which can be interpreted by the combined effect of lateral motion and intrachain rotational isomerism.

STATISTICAL PHYSICAL MODEL OF THE PHASE TRANSITIONS OF LECITHIN MEMBRANES

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In the recent years a great number of papers has dealt with the phase behaviours of synthetic lipid membranes made from phosphatidylcholines. It is well known, that some of them like dipalmitoyl-phosphatidylcholine, distearoyl-phosphatidylcholine have three phase transitions, called main-, pre- and subtransition. Our model deals with the latter two ones.

A statistical mechanical model has been worked out to explain the pre- and subtransition of the artificial lipid membranes, which model is based upon some experimentally proved facts. The orientation of chains being "all trans" state and the interaction between the electrical dipols of head groups are taken account.

Our model is able to describe the great hysteresis of the subtransition, which was found in DSC and NMR measurements. Using the model it can be proved that this is a consequence of the coupling between the head groups and chains of lipid molecules. We can explain the packing of chains, the ordering of head groups, and mobility changes at both phase transitions. These results are in good agreement with the NMR measurements available in the literature.

Applying the model to lipid molecules of different chain length, a dependence of the transition temperature on the chain length has been obtained. The trend of this dependence is the same as that of the experimental results.

THE EFFECT OF GLYCOSAMINOGLYCANS ON LOW-DENSITY LIPOPROTEIN /LDL/. INVESTIGATIONS WITH MODEL MEMBRANE

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LDL plays a very important role in the pathogenesis of atherosclerosis. An important step in the atherogenic process is the interaction of LDL with arterial glycosaminoglycans /GAG-s/. The complex formation is mediated by calcium ions. As it was published earlier /Bihari-Varga/ the GAG-LDL interaction alters significantly the phase transition temperature of LDL.

In our experiments the phase transition of dipalmitoyl--phosphatidylcholine /DPPC/, one of the main components of LDL was measured. From the GAG-s, chondroitin sulfate-6 /CSA-6/ was investigated which is the main GAG component in the arterial wall. The measurement was performed by differential scanning calorimetry /DSC/ technique /DuPont 999/.

In the first part of the experiments at a given /5:1/ weight ratio of DPPC to CSA-6 we changed the amount of Ca-ions. A saturation curve was obtained with a saturation point at 0.15 weight% Ca-ion to DPPC. Next we changed the DPPC to CSA-6 ratio, fixing the amount of Ca-ion at 0.2 weight% and at 0.05 weight% in the two series of experiments, respectively. The curves resembled the previous one. The saturation point was at about 4 weight% CSA-6 to DPPC.

Conclusions:

1/ Ca-ions are needed for the formation of the complex.

2/ The complex formation is limited by the component present in the lowest concentration in the solution.

3/ The optimal DPPC:CSA-6 disaccharide subunit:CA molar ratio was found to be 180:12:5.

INTERACTION OF NONIONIC TENZIDES WITH PHOSPHOLIPIDS STUDIED BY "CHARGE TRANSFER" CHROMATOGRAPHY

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Thin-layer chromatographic plates prepared from mixtures of diatomaceous earth:Kieselgel G 2:1 weight ratio were impregnated by 5% paraffin oil in h-hexane. Dipalmitoyl phosphatidyl choline /DPPC/, dioleyl phosphatidyl choline /DOPC/ and dipalmitoyl phosphatidyl ethanolamine /DPPE/ were chromatographed in eluent water:i-propanol 11:9 vol. ratio. Nonylphenyl-ethylenoxide, tributhylphenyl-ethylenoxide polymers, ethoxylated oleic acid derivatives, Tween 20, 40, 60 and 80 were added to the eluent in different concentrations. The nonionic tenzides forming complexes with phospholipids decreased their lipophilicity, the degree of lipophilicity decrease was related to the complex stability.

Each tenzide formed complexes with each phospholipid. The complex forming capacity of nonylphenyl and tributhyl-phenyl-ethylenoxide polymers did not depend significantly on the number of ethylenoxide groups in the tenzide molecule. The hydrophobic part of tenzides influenced considerably the strength of interaction. In each case the complex forming capacity of DPPE was significantly higher than those of DPPC and DOPC proving that the head groups of phospholipids have a preponderant role in the complex formation.

MOTION OF THE PURPLE MEMBRANE DURING THE PHOTOCYCLE OF BACTERIORHODOPSIN

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Bacteriorhodopsin is the only protein contained in the purple membrane of Halobacterium halobium. As a part of the cell membrane, it performs a protonpumping function via a photochemical cycle whose steps are identified using absorption spectroscopy and flash photolysis. Spectroscopic measurements on purple membrane are disturbed by the light scattering of the particles. Systematic study of the scattered light leads to the observation that scattering changes during the photocycle, and that this change depends on the flash polarization, pH, and the solvent viscosity. A theoretical model is given to explain the experimental data. The model asserts that the purple membranes are bent and, during the photocycle, the degree of bending varies due to the conformational change of the bacteriorhodopsin molecules.

THE STOICHIOMETRY OF THE BACTERIORHODOPSIN PHOTOCYCLE AND "PROTONCYCLE" IS NOT CONSTANT: IT IS REGULATED BY $\Delta\tilde{\mu}_{H^+}$

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An important parameter of active ion pumping systems such as bacteriorhodopsin /BR/ and electron transport chains in chloroplasts and mitochondria is the stoichiometry /S/: the number of ions pumped per one turnover of the system. For BR it was recently proved that the rate of the photocycle is controlled by $\Delta\tilde{\mu}_{H^+}$ /electrochemical potential of protons/.

In this study we provide experimental evidence for the control of $\Delta\tilde{\mu}_{H^+}$ on S. For this, the kinetics and the amplitudes of the pH changes and the M_{412} intermediate decay were studied in Halobacterium halobium cell envelope vesicles in 4M NaCl at pH=7. The absorbance changes of BR and a pH sensitive dye /pyranine, 80 μM/ upon flash excitation / ∿2 μs/ were determined in a flash-photolysis setup / λ=450 nm/. The difference in the M-decay curves between two identical samples, but with and without pyranine was used to determine the ΔpH generated by BR. Increasing levels of $\Delta\tilde{\mu}_{H^+}$ were generated by continuous green background light illumination from a Ar-ion laser. Results:

1. At increasing background light intensities more and more M formes in steady state. Consequently, the flash produces less M. Accordingly, ΔpH generated by flash light is smaller in the presence of background light.
2. However, the drop in the pH signal is much mor pronounced than the decrease in M-formation. Thus the H^+/M ratios measured /S/ at increasing $\Delta\tilde{\mu}_{H^+}$ decrease gradually.
3. We propose that the increasing ΔΨ favours a "non-pumping" branch in the photocycle of BR which may have a regulatory role.

MEASUREMENT OF MEROCYANINE 540 LATERAL DIFFUSION IN THE CYTOPLASMIC MEMBRANE

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MC540 is a potential-sensitive, hydrophobic stain readily incorporating into the fluid domains of cellular membranes. In this communication we report the results of an analysis by flow cytometry and fluorescence recovery after photobleaching /FRAP/, of MC540 staining of mouse spleen cells. We show that 1./ UV illumination of the cells before the addition of the dye significantly enhances staining; 2./ UV pretreatment causes an increased permeability toward propidium iodide, Hoechst 33342, and fluorescein, as well; 3./ the increment in MC540 fluorescence precedes permeabilization to propidium iodide; 4./ the event of permeabilization can be visualized under fluorescent microscope or in FRAP experiments; 5./ at room temperature the lateral diffusion coefficient of MC540 is about 1×10^{-8} cm^2/s; 6./ this value markedly diminishes upon lowering the temperature or glutaraldehyde fixation of the cells.

ON THE ROLE OF THE INTERFACIAL WATER; THE HYSTERESIS OF L_p

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The importance of water ordered on colloid surfaces is more and more appreciated both in biotechnics /e.g. membrane separating methods, tissue compatibility, inhibition of lithiasis and sclerosis, membrane fusions, the snow producing effect of Pseudomonas syringae, freeze resistance, water retention of muscle etc./ and in biological processes /e.g. activity and conformation, oedemas, antibiotics, anesthetics, membrane channels etc./.

If in the pore water of cellophane membrane phase transition occurs depending on temperature, a characteristic change of water permeability /L_p/ can be expected. The outflow from water into water out of a cellophane sack of about 10 cm^3 and held up by a gauze net was measured as a function of temperature at a constant hydrostatic pressure difference. It corresponded to the temperature dependence of fluidity but between 10-33°C and 43-50°C left-handed and between 33-43°C right-handed hysteresis is obtained. This direction-dependent deviation is 5-10 % in the value of L_p and appears only under a certain L_p threshold value /pore size/. It was not demonstrable by ethanol, glycerine and DMSO on the cellophane, but it could be demonstrated by water on the PVC membrane, too. The conceptual importance of it - if it is proved later - is that the /metastable, dissipative?/ water domains arranged on surfaces raise the possibility of "molecular memory".

THE BIOLOGICAL EFFECT OF THE HeNe LASER RADIATION ON THE HUMAN SKIN OF THE FACE USED IN POINT-LIKE AND AREA-LIKE APPLICATIONS

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MEDICOR Company, H-1389 Budapest

The HeNe laser called LASERMED /manufactured by MEDICOR/ was applied by the authors. This instrument has a 5 mW output power with polarised light at 632.8 mm wavelength.

In the cosmetical practice the affected areas are localized mainly on the face. On 13 volunteers the point-like and area-like applications were studied, in the latter with special respect to the direction of the "beak-lines" of the skin.

Before and after radiation the biological response was followed by measuring skin respiration, skin temperature and the electrical skin resistance locally /on the place of the radiation/ and non-locally /at a distant place/. The results were evaluated by linear discriminant-analysis.

Conclusions:

1. The effects of the point-like and the area-like applications are equally significant.
2. The laser-effect is not restricted only to the spot of radiation.
3. In the case of elder patients the laser-treatment proved to be more effective than in the younger generation / 37/.

COMPARISON OF TUMOR CELL LINES OF DIFFERENT METASTATIC ABILITIES APPLYING SPECTROSCOPIC METHOD

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Two Lewis lung tumor cell lines were compared, using ESR /electronspin resonance/ spectroscopy. For the ESR experiments the prepared cells were suspended in PBS-solution. In the experiments the fluidity of the lipid region, the mobility of the peripherial proteins, and the reduction ability of the cell lines were compared.

The fluidity of the lipid phase was probed by the spin labelled fatty acids, 5-doxyl- and 16-doxyl-stearic acid derivatives, at two different depths relative to the lipid head groups.

Our results show that a fluidity difference can be detected at the vicinity of the polar head group along the hydrocarbon chain; the fluidity is smaller in the cell line of greater metastatic ability.

Probing the peripherial proteins with spin labelled maleimide derivative, it was concluded that there is a significant difference between the two cell lines. The mobility of the peripherial proteins and/or the hydrophobicity of the nearest neighbourhood of the monitor group on these proteins is smaller for the cell line with less metastatic ability.

Measuring the reduction ability of the cell lines, it was observed that the reduction ability of the cells of greater metastatic ability is about four times smaller than that of the cell line with smaller metastatic ability.

DISTRIBUTION OF ANIONIC SITES ON SURFACES OF VARIOUS B_{16} MELANOMA CELL LINES

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The distribution of surface negative charges of three B 16 melanoma cell lines metastasizing differently were studied by electronmicroscopy in situ using cationized ferritine /CF/. In all three B 16 melanoma clones examined the CF binding sites were more expressed on apical plasma membrane regions, than on basal ones. The polarity of CF binding to cell membranes as ratio of percentual coverages on apical and basal surfaces in the poorly metastasizing "18" clone was 5.8 and this value reflected that at basal localization no CF binding could be observed except for the cell-to-cell junction areas. The distribution of CF on the apical region was uniform.

In cell clones of high metastasizing capacities, "10a" and "12/2" clones the extents of polarities were less, i.e. 1.2 and 1.6, respectively. This was manifested in well detectable distribution of binding sites between the apical and basal surfaces, though no difference was observed in the percentual, coverage of the total surfaces. On these cell clones the CF was localized in clusters.

These observations suggest a possible correlation between the polarization of surface charges and the degree of metastatic potential of various tumor cells.

CHARACTERIZATION OF THE PHOTOSYNTHETIC ELECTRON TRANSPORT CHAIN IN NORMAL AND PHOTOBLEACHED ANABAENA CYLINDRICA BY FLASH SPECTROSCOPY

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Electron transport of normal and photobleached Anabaena cylindrica was studied using spectral and kinetic analyses of absorbance transients induced by single turnover flashes. Between 500 and 580 nm two positive bands (~540 nm; ~566 nm) and two negative bands (~515 nm; ~554 nm) were found. Absorbance changes at 515 nm and 540 were partly characterized. None of these absorbance changes represent an electrochromic shift. Absorbance changes at 554 nm and 566 nm correspond to the oxidation of cytochrome f and the reduction of cytochrome b_{563}, respectively. We found a very slight 3-(3,4-dichlorophenyl)—1,1-dimethylurea (DCMU) sensitivity of cytochrome f in normal cells, while DCMU was completely ineffective for cytochrome f reduction in photobleaced cells. The absorbance change of cytochrome b_{563} increased, while absorbance change of cytochrome f was smaller than in normal cells. The increased O_2 evolution in photobleached cells and the negligible electron transport via cytochrome f suggest the participation other electron acceptors in the electron transport chain of photobleached Anabaena cylindrica.

MERIDIANS EXPLAINED BY NEUROBIOLOGICAL RESULTS

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Presuming that acustimuli are propagated primarily by nervous media, the real question is why from the acupoints only and not from any other point of the body surface the beneficial effects can be elicited?

In the explanatory model, point-to-point /PTP/ type projections from the peripherial points and from the inner organs to the spinal cord, as well as a many-to-many /MTM/ type projection from the spinal cord to the spinal cord itself /realized by the system of the spinospinal fibers/ are considered.

So, by the PTP projection the surface of the body and the inner organs are mapped onto the spinal cord in superimposition; and by the MTM projection, with ideal fibers, a homogeneous focusing phenomenon would take place all over on the spinal cord.

Since evolution could not be involved, the so-called intrinsic acupoints in the spinal cord are considered to be failure places of the ontogenetic development of the fiber systems of the spinal cord. Functionally the intrinsic acupoints are "hole"-s in the homogeneous focusing phenomenon, and the specific fibers connecting intrinsic acupoints to each others are the embodyments of the meridians. Consequently, the proper fibers of PTP only connect acupoints to intrinsic acupoints.

The lower resistance at the acupoints is thought to be a result of higher perspiration locally; as an effect of the inequilibrium between the sympathetic and parasymphathetic innervation caused by the "hole"s.

PSORALEN DERIVATIVES-INDUCED STRUCTURAL CHANGES IN NUCLEOPROTEINS

K. Tóth, J. Fidy, K. Fehér

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Structural aspects of a naturally occurring and therapeutically used photochemical reaction were studied in order to study the molecular changes of biological consequences.

DNA-containing T7 phage and RNA containing MS2 phage nucleoproteins /NP/ as well as isolated nucleic acids /NA/ were examined as biological targets. The chemical agents were psoralen-derivatives, active under near-UV irradiation. The dose- and wavelength-dependence of the irradiation was tested.

The dark and photoreaction of the psoralen-NP or-NA systems were examined in different buffer solutions from two points of views: the fluorescence intensity and spectral changes report about the amount and state of the free or bound psoralen molecules, the optical melting temperatures and cooperativity indices indicate the modifications of structure of NP and NA.

Results: Dark complexes are formed through electrostatic and stacking forces. Using the model of McGhee and van Hippel /1/ to determine the binding constants, a decreasing effectivity can be shown in terms of RNA, MS2 phage, DNA, T7 phage, for 4'-aminomethyltrimethylpsoralen as agent. High ionic strengths (>0.1) or the presence of divalent cations (~1 mmol/l) hinder the dark-binding. The photoreaction is a two-step process. The first covalent binding is formed under irradiation of either small doses (<1 kJ/m^2) at 340 nm or of any doses at around 370 nm. This first step leads to the destabilization of the NA helix. A fluorescent photoproduct is to be found. The second step is a binding of the psoralen on both ends to NA in cases when the further irradiation takes place at shorter wavelengths (~340 nm). The stabilization of the helix structure

of NA is proportional to the UVA dose. The protein denaturation in NP-s is also influenced by the photochemical reaction.

/1/ McGhee J.D., van Hippel P.M., J. Mol. Biol. 86 /1974/ 469.

THE EFFECT OF FUROCOUMARINE DERIVATIVES ONTO RNA-PROTEIN COMPLEXES

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Biophysical Institute, Semmelweis Medical University, H-1088 Budapest

Photochemotherapeutic agents, like furocoumarine derivatives are widely used in the psoriasis therapy. One of the most powerful synthetic compounds of this group is 4'-aminomethyl-4,5',8-trimethylpsoralene /AMT/. The AMT-DNA and AMT-DNA-protein interactions have been investigated in the literature. We have studied the influence of the AMT binding on the structure and biological activity of an RNA-protein complex; the MS2 bacteriophage and its isolated RNA, in the presence and absence of light.

The process of AMT binding can be studied by fluorescence spectroscopy based on AMT fluorescence. AMT kept in dark was found to interact strongly with isolated RNA as well as its protein complex in MS2 bacteriophages. The values of association constants were significantly higher, in comparison with those of DNA-protein complexes. On the other hand, the qualitative and quantitative features of AMT binding exposed to light were found to be dependent on the wavelength of the excitation light.

The binding effect on the biological activity of bacteriophages treated with AMT solutions was investigated by the method described earlier /1/. In this study 10^{-4} mol/l AMT solutions were used and the plaque forming ability was checked in dependence of incubation time. The effect of AMT solutions either exposed to light at 370 nm for 3 hours or kept in dark before adding the phages was compared. The incubation was carried out in dark at room temperature for 1, 2 and 3 hours. We found that the AMT solution exposed to light significantly decreased the multiplication capability of bacteriophages, while the AMT solution, prepared freshly and kept in dark had no

similar effect onto MS2 phages. Thus, the possible mutagenic side effects of this therapeutic treatment must be considered.

/1/ Gy. Rontó: Bakteriofágok és részrendszereik pontszerü sérülése, D.Sc. Thesis, Budapest, 1979.

COMPARATIVE LUMINESCENCE STUDIES ON THE INTERNAL DYNAMICS OF DEPHOSPHORYLATED AND PHOSPHORYLATED FORMS OF GLYCOGEN PHOSPHORYLASE

I. Seres, B. Somogyi, J. Matkó

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Nowadays the biochemical and structural differences between phosphorylated /a/ and dephosphorylated /b/ forms of glycogen phosphorylase are of special interest with respect to catalytic and regulatory mechanism of this enzyme. The very effective X-ray crystallographic method, however, has been limited so far to the inactive T form of the enzyme. At the same time, spectroscopic methods were able to show numerous transient conformations in solution. These facts and the difference in the oligomeric structures of a and b forms necessitate detailed comparative studies of conformational dynamics in solution.

First we studied the overall motility of the protein matrix in the two forms by fluorescence techniques. Förster-type resonance energy transfer /Trp→PLP/ and quenching of TRP emission were used to investigate internal dynamics of the protein structure. The covalently activated a form showed a reduced overall motility as compared to the nucleotide activated b form on the basis of activation energies obtained for the quenching by acrylamide. Results of temperature-dependent energy transfer investigations led to the same conclusion. Coenzyme PLP fluorescence quenching experiments also reported differences between the two enzyme-forms.

TRANSIENT ABSORPTION AND EMISSION OF SCHIFF-BASE COMPOUNDS AND THEIR APPLICATION TO STUDIES ON ROTATIONAL MOBILITY OF PROTEINS

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Slow rotational motions of membrane-bound proteins or nucleic acids are of special interest in cell-biophysics, since characteristic parameters of these motions may reflect interactions and orientations of these macromolecules as well as the properties of their environment. The methods developed so far to study these motions /e.g. phosphorescence anisotropy, absorption dichroism/ are based on the use of different triplet probes. We have shown that PLP- or salyciliden complexes of proteins undergo excited state proton —transfer reaction forming a long-lived /μs-ms/ metastable singlet state. The latter shows characteristic transient-absorption and fluorescence whose lifetime and intensity are very sensitive to environmental polarity, therefore the method is applicable to study local conformational changes of proteins, as well. They are, however, not sensitive to the presence of oxygen, which eliminates the technical problems coupled to oxygen--sensitivity of triplet probes. Using polarized laser beams, detection of anisotropy decay of the transient fluorescence of these probes allows an advantageous determination of rotational relaxation times for membrane bound proteins as well.

LIGHT INDUCED PICOSECOND CHARGE MOVEMENT IN PROTEINS

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Recent developments in the generation of picosecond and subpicosecond laser pulses made possible the study of ultrafast processes in proteins. Primary charge separation in photosynthesis, light energy transduction in rhodopsin and bacteriorhodopsin, photodissociation of ligands in heme proteins, etc. were found to take place in picosecond time scale. Though all the above processes include some charge movement, they were investigated generally by pure optical methods. Here we are presenting an experimental technique suitable for the direct electrical detection of picosecond charge motions in oriented biological samples. The time resolution of our method is by an order of magnitude greater then that of any previous one. The method was applied for the study of the primary charge separation in the purple membrane of bacteriorhodopsin.

Dried oriented purple membrane samples were mounted onto a home-made 50 ohm coaxial electrode system. The samples were excited by the 55 ps pulses of a distributed feedback dye laser. The electrode system was directly connected to the sampling head of a 14 GHz sampling oscilloscope /rise time 25 ps/. The output signal of the oscilloscope was digitalized and averaged in a multichannel analyzer. Computer simulation of the response of the measuring system to an exponentially decaying charge motion inside the sample was carried out for different decay constants. Comparing the theoretical curves with the experimental ones we found the best fit at a time constant of 30 ± 10 ps. This correlates well with the optically measured 11-16 ps formation time of the K intermediate of bacteriorhodopsin photocycle. Since optical measurements

predict even faster charge movements a faster measuring system is under construction in our laboratory.

THE MECHANISM OF PROTON CONDUCTION IN LECITHINS

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In the experimental support of the application of semiconduction concept in biology an important role has been given to the electrical conduction studies. However, measurements proving unambiguously the electronic nature of conduction in hydrated biological materials, particularly in lipids, have not been conducted. Therefore, the experimental proof of the semiconductor behaviour of lipids can not be considered as completed.

To establish the nature of conduction we undertook imped ance measurements, electrolysis and thermoelectric investigations on lecithins at different hydration levels and temperatures. It was found that the conduction was not electronic, but protonic in nature.

From the results of the thermoelectric measurements the energy of proton generation was estimated. The energy of charge generation did not depend on the number of adsorbed water molecules but it was dependent on the phase state of the lipid. It was smaller in the gel state, around 15-19 kJ/mol, and 35-37 kJ/mol in the liquid-crystalline state. The process of charge generation can be explained by the interaction of water with the phosphate group of the lecithin molecule. From the activation energy of conduction the activation energy of charge mobility was also estimated. Its value varied in a wide range depending on the water content, and it was influenced mainly by the mobility of the lecithin head groups. According to our view, structure units consisting of several water molecules are involved in the conduction process.

LIGNOCELLULASE PRODUCTION ON AGRICULTURAL WASTES

K. Zetelaki-Horváth, G. Vereczkey, M. Németh

Central Food Research Institute, H-1022 Budapest

White rot fungi are considered to be good lignocellulose degraders, but different species decay the various components of lignocelluloses at different rates. As Phanerochete species preferentially degrade lignin to cellulose, this organisms were chosen in our work for the production of lignolytic enzyme, using lignocellulose-containing agricultural wastes as substrate.

For the enzyme production a special solid-continuous fermentation method was been used, developed /Zetelaki-Horváth, 1984/ for this very purpose previously. A tubular fermentor was filled with ground cornstalks, completed with bios materials and minerals and was inoculated by shaken culture of Phanerochete chrysosporium /ATCC 32629/. After colonization of the substrate by the organism, addition of mineral solution has been started at flow rates of 50 cm^3/h and 600 cm^3/h in the 6 and 40 dm^3 fermentors, respectively. The continuously collected effluent had rather high enzyme activities (filter paper /FP-ase/, carboximethylcellulase /CMC-ase/, lignolytic activity /LC-ase, expressed as lignin loss of straw after treatment/).

Results obtained in the first and the final parts of the cultivation in the 6 and 40 dm^3 fermentors:

Volume dm^3	Cultivation period (h)	Fp-ase (mg/cm^3)	CMC-ase ($dm^3/h.dm^3$)	LC-ase (%)
6	67-45	2.94	15810	28.0
	145-290	1.86-0.86	8694-5690	32.4-40.0
40	95-169	4.40-3.53	25060-13460	0.6-1.6
	220-313	0.88-0.59	4111-3266	21.0-24.0

The activity of lignocellulase has been studied on native straw and craft lignin /Indulin AT/ substrates.

LIGNIN DECOMPOSITION BY LIGNOLYTIC ENZYME COMPLEX

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It is well known that the digestibility of lignocelluloses can be increased by decreasing their lignin content.

The aim of our work was to elaborate a method for the determination of the lignin decomposing activity of the enzyme complex produced at the Bioengineering Department by Phanerochete chrysosporium, using a special solid continuous fermentation /Zetelaki-Horváth, 1984/.

The lignin decomposition by the enzyme complex was determined by the decrease in lignin content of straw and cornstalks before and after enzyme treatment. The sodium chlorite /Collings et al., 1978/ and the Van Soest /1963/ methods were used, and their results compared. Lignin determination was continued by instrumental method, using the automatic Fibertec equipment /Tecator, Sweden/, which increased the reliability of the determinations.

Lignin decomposition of the enzyme complex on natural straw varied between 15-30% /incubation: 24 h at 30°C/.

For lignin determination the near infrared reflectance technique was also tested by an INFRAPID 61 equipment /LABOR MIM, Budapest/, when correlation coefficient between the sodium chlorite lignin and the physical parameters proved to be 0.93.

A spectrophotometric method has been developed for the activity measurement of the lignolytic enzyme complex, using craft lignin /Indulin AT/ as substrate. After 24 hour incubation a lignin decrease of 5-25 % was found using a 0.5 per cent Indulin suspension and an enzyme concentration of 0.5-2 per cent.

COMPUTER MODELING STUDIES FOR PROGNOSTICATION OF THE EFFECTIVENESS OF STABLE IODINE PROPHYLAXIS

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The aim of our computer modeling studies was the elaboration of a way of exact prognostication of the effectiveness of stable iodine prophylaxis used to prevent thyroid gland radiation damage resulting from accidental radioiodine incorporation. We studied if our method, i.e. the program we developed according to Johnson's modified version[1] of Riggs iodine kinetics model, adequately permitted modeling of iodine metabolism perturbed by application of stable iodine depending on the average daily iodine intake and scheme of iodine prophylaxis. Decrease of thyroid gland radiation burden was calculated on basis of residence times as determined by the program. Our results prove that the protective effect of stable iodine prophylaxis was less in population living in areas of lower iodine supply. While the radiation burden of thyroid gland increased with the elevation of the interval between radioactive and stable iodine application, with the increase of iodine doses applied at the same intervals, thyroid gland dose decreased only slightly. Our data support the results[2] of human experiments, so we think our program suitable for the prognostication of the efficiency of stable iodine prophylaxis.

1. Johnson J.R., J. Radioanal. Chem. 1-2. 223. 1981.
2. NCRP, Report No. 55, Washington D.C. 1977.

TEACHING BIOPHYSICS AT THE RADNÓTI MIKLÓS GRAMMAR SCHOOL

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[1]Radnóti M. Grammar School, [2]Biophysical Institute, Biological Research Center of the Hungarian Academy of Sciences, [3]Biochemical Institute, Medical University, H-6701 Szeged

At our grammar school biophysics teaching began in the autumn of 1981 in the form of a study circle. In the first year it was open for 3rd year pupils. The interest was very great. This group was acquainted with the elementary mechanisms of excitations, the biophysics of sensation and the biomechanics of muscles.

In the next school-year we went on with this group and also a new group was formed for the 3rd year pupils of that academic year. One of the biology and chemistry teachers of the school then received support from the Hungarian Academy of Sciences to carry on his research, for a year. He began dealing with the second study circle and developed a collaboration with the biophysical laboratory of the Biochemical Institute of SZOTE. At the 4th year, too, the study program of these pupils was supported by this laboratory. This group paid visits to academic and university institutes that year.

By now, we have some experience concerning all the four years of grammar school. We seem to have succeeded - with minor difficulties - in selecting certain parts of biophysics for all the four forms that the pupils with their knowledge are able to integrate in the material of their biology, physics, chemistry and mathematics subjects.

Our experiments were supported by the Biochemical Institute of SZOTE through the deputy rector of that university, the Biophysical Institute of the Biological Research Centre, and the Chairman of the Hungarian Biophysical Society.

After four years of experiments the Radnóti M. Grammar School now has arrived at the stage when, by a directive of the Ministry of Education, it can open a half form in biophysics for the school year 1985-86 within the framework of

ordinary daytime education. We are over with the entrance examinations. From the number of applicants we came to the conclusion that by starting a special class like this we meet a considerable demand of the society.

AUTHOR INDEX

Biological Chemistry Hoppe-Seyler

Edited by K. Decker, W. Stoffel, H. G. Zachau

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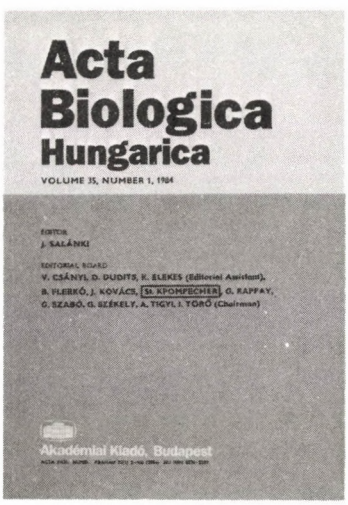

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1828

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Foreword

This issue of Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae is dedicated to Professor Bruno F. Straub on the occasion of his 70th birthday, celebrated on January 5, 1984.

F. B. Straub's life and works span several epochs of international and Hungarian biochemical research. He started his career at Szeged University, in Albert Szent-Györgyi's laboratory, where he read medicine for three years, then switched over to chemistry. After another two years he graduated as biochemist, an unprecedented procedure then at the university, carried out under Szent-Györgyi's influence. Contemporary military authorities did not accept his doctorate and gave him the low rank of corporal when drafted. However, Corporal Straub rapidly rose, due to diligence and virtue, to the rank of general, though not in the military but rather in the scientific field.

He became full professor of biochemistry and member of the Hungarian Academy of Sciences at a poetically young age (32). And, in an enigmatic manner, he somehow maintained the image and stature of the "young professor" even in his advanced years. He is the live example that youthful vigour and endurance of the mind are gifts hardly age-dependent. At 70, in a sense he is younger than many of his disciples junior to him by a whole generation.

In 1960 he was appointed director of the Institute of Biochemistry of the Hungarian Academy of Sciences. His activity was instrumental in the foundation of the Academy's Biological Research Center in Szeged, so far the greatest enterprise in experimental biology in Hungary. He served the first two terms (1970 to 1978) as Director General at this Center shaping it into a scientific stronghold of international reputation. The list of honours, distinctions, etc. bestowed on him would take more space than allotted to this whole foreword.

What are then Straub's outstanding scientific achievements? The answer should be given, I deem, from two viewpoints: international and national. Young biochemists abroad know him mainly after his early work: the discovery of actin, Straub's diaphorase (lipoic dehydrogenase), crystalline lactate dehydrogenase, ATP-dependent K^+ accumulation of red cells, each representing a landmark in the respective areas. Senior biochemists abroad also know him as science diplomat, member, chairman, etc. of numerous top international bodies. For us Hungarians, he is all this and more. He is the man who after World War II stayed in this devastated country while many weighed anchor and sought milder

Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae 20, 1985
Akadémiai Kiadó, Budapest

harbours. He upheld scientific standards during harsh and lean years. It is perhaps highly unusual, when celebrating him, to speculate how much more this talented biochemist could have accomplished in a more affluant ambience. However misplaced this question may appear in a jubilee note, it bears on a crucial point in Straub's service to his country. For the world's biochemistry perhaps suffered some loss by his remaining on board here, but we Hungarian biochemists certainly gained a lot by his presence. It is this we appreciate in him most.

The small bunch of papers included in this issue represents but a fraction of current biochemical research trends in Hungary initiated by Straub, his co-workers and pupils. Let it be a modest salute enwrapping the wish that he shall remain the active witness of our endeavours for many years to come.

Peter Friedrich

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Is Elastomucoproteinase a Double-Headed Enzyme?

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(Received February 25, 1984)

During isolation pancreatic elastase is accompanied by a minor component, elastomucoproteinase (EMPase), which can be separated in pure state by anion-exchange chromatography.

EMPase exhibits both proteolytic and mucolytic activities. Applying powdered aortic preparations as a substrate, the enzyme can split off carbohydrate moieties, but it can also cleave peptide bonds as a proteinase.

Assaying with tripeptide-p-nitroanilide substrates, the enzyme can decompose only those substrates which contain Phe or Tyr at the C-terminal. It appeared that the binding of Bz-Ala-Gly-Phe-pNA was the best, while the hydrolysis of Bz-Ala-Pro-Tyr-pNA was the fastest.

Proteolytic activity of EMPase can be completely inhibited with phenylmethanesulphonyl fluoride, whereas its mucolytic activity only by 20%. The two activities may be located at two separate sites. The enzyme seemed to be composed of a single polipeptide chain by SDS-gel electrophoresis in the presence of urea and β-mercaptoethanol.

Pancreatic elastase was isolated first by Baló and Banga (1949). Later it became evident that the preparation consists of two principles at least, i.e. proteolytic E_1 and mucolytic E_2 components (Hall, 1955). Then it was found that elastomucoproteinase, collagen mucoproteinase (Banga and Baló, 1956) and elastolipoproteinase with lipolytic activity (Banga and Baló, 1962; Banga 1966) are strongly associated with the partially purified elastase.

According to several authors, elastomucoproteinase is usually present in the elastase preparations (Hall, 1957; Loeven, 1960; Hall and Czerkawski, 1959), but as it could be isolated only in small quantities, the properties of the enzyme were not described in details. The two enzymes can be separated by adsorption on γ-alumina gel (Hall, 1957; Hall and Czerkawski, 1959), or by DEAE cellulose (Lewis and Thiell, 1957) and DEAE-Sephadex chromatography (Loeven, 1963). The purified enzyme was found to cleave acetyl-Tyr-ethylester, the substrate of chymotrypsin (Ardelt, 1974).

In the presence of EMPase, elastase exerts increased elastolytic effect (Banga and Baló, 1956). Elastase is able to decompose the polypeptide chain of elastic

Abbreviations: EMPase, elastomucoproteinase; Suc-, succinyl; Ac-, acetyl; Bz-, benzoyl; Z-, benzoyloxycarbonyl; -pNA, p-nitroanilide; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl; BTEE, N-benzoyl-L-tyrosine ethyl ester; NBA, N-tert-butoxycarbonyl-L-alanine-p-nitrophenyl ester

fibres effectively only when the polysaccharide substituents had previously split off (Banga, 1966).

In the present paper the isolation of EMPase in sufficient quantities is described. Besides, the multifunctional properties of the enzyme, i.e. amidolytic, esterolytic, mucolytic and proteolytic activities were also investigated and the substrate specificity was determined.

Materials

Chemicals were obtained as follows: NBA, BTEE, bovine serum albumin, carbazole from Sigma Co. (St. Louis, Mo., USA); Ac-Tyr-pNA, Suc-$(Ala)_3$-pNA, proteins for molecular weight determination and PMSF from Serva (Heidelberg, FRG); DEAE-Sephacel from Pharmacia Fine Chemicals (Uppsala, Sweden); casein and papain from Reanal (Budapest, Hungary).

Crude elastase and aorta powder were obtained by the method of Banga (Banga and Baló, 1962; Banga, 1968, 1969). Denatured haemoglobin was prepared from beef red blood cells (Anson, 1938). Elastine was isolated from beef nuchal ligament according to Partridge and Davis (1955).

Tripeptide-pNA substrates having Phe or Tyr at the C-terminal were generous gifts of Dr. L. Aurell (KABI, Mölndal, Sweden). The other peptide substrates were synthesized in our laboratory. The homogeneity of the products was analysed by thin-layer adsorption chromatography, their composition was controlled by elementary analysis (Cs.-Szabó et al., 1980).

Methods

Protein concentration at various steps of isolation was determined according to Lowry et al. (1951) using bovine serum albumin as reference.

Gel electrophoresis

The purity of EMPase was controlled by slab technique on 10% polyacrylamide gel. To $82 \times 82 \times 2.3$ mm gel 6–50 μg protein was applied and run in 40 mM β-alanine-acetic acid buffer, pH 4.5, at 6 V/cm for 30 min, then at 12 V/cm for 3.5 hrs. The slabs were stained for protein with 0.1% Coomassie brillant blue R-250 dissolved in methanol-acetic acid-water (7 : 3 : 1).

To determine the molecular weight (Laemmli, 1970), proteins were pretreated by boiling for 5 minutes in 2% SDS and 5% β-mercaptoethanol, then run in 5–15% acrylamide gradient gel containing 0.1% SDS, 1 M Tris buffer, pH 8.8, at 20 mA/50–200 V. Molecular weight references: ribonuclease (13 600), horse myoglobin (17 800), chymotrypsinogen (25 000), ferritin (36 400), egg albumin (45 000) and bovine serum albumin (67 000).

Enzyme activity with synthetic substrates

Enzyme activities with synthetic substrates were assayed spectrophotometrically at 37 °C and the changes in absorption were recorded.

a. A 0.01 change of absorption in 1 min was taken as a unit of esterase activity. Elastase activity was determined with NBA substrate, in 50 mM phosphate buffer, pH 6.2. The final concentration of the substrate was 50 μM. The increase of absorption was recorded at 347.5 nm.

The activity of EMPase was measured at 256 nm with BTEE in 20 mM Tris-HCl buffer, pH 8.2. Substrate concentration was 50 μM.

b. Amidolytic activity was examined at 405 nm with tripeptide-p-nitroanilide substrates in 20 mM Tris-HCl buffer, pH 8.0, containing 4 mM $CaCl_2$. Concentration of the enzyme varied between 1 – 100 nM, that of the substrate between 10–100 μM in a final volume of 1.5 ml. The increase in absorption of the liberated pNA was followed at 405 nm ($\varepsilon_{405} = 10\,600\ M^{-1}\ cm^{-1}$).

Kinetic constants (K_m, V_{max}) were calculated according to Lineweaver-Burk, while catalytic constants, k_{cat}, from the $V_{max}/[E]$ ratio.

Proteolytic activity

Haemoglobin, casein and elastin substrates in 20 mM Tris-HCl buffer, pH 8.0, containing 4 mM $CaCl_2$ were applied for the determination of proteinase activity. The enzyme concentration was 0.16 μM and the substrate concentration was 2%. After 60 min incubation equal volume of 10% TCA was added and the amount of soluble peptides was determined with biuret reagent.

Mucolytic activity

Three ml suspension of 1% aorta powder in 20 mM Tris-HCl buffer, pH 8.0, containing 4 mM $CaCl_2$, elastase or EMPase in 0.1 μM concentration was incubated for 24 hours. Then a mixture of 2 ml 31% NaCl and 100 μl 3N acetic acid was added, it was boiled and centrifuged. The mucopolysaccharides in the supernatant liberated upon mucolytic effect were precipitated with 96% ethanol containing 0.08% sodium acetate. The precipitate was dissolved in distilled water, and hexuronic acid content was determined (Dische, 1947). Glucuronic acid was used as reference. Total mucopolysaccharide content was liberated by papain digestion and was determined under the same conditions.

Results

Isolation of EMPase

Crude elastase of 1.5 g (protein content: 650 mg) was extracted twice, with 15 ml and 30 ml 500 mM acetate buffer, pH 4.5, containing 30% ethanol, at 4 °C, stirring for 30 min. Then it was centrifuged (10 000 r.p.m., 30 min, at 4 °C) and the supernatants were collected while the precipitate was discarded. This extract

Table 1

Isolation of EMPase from crude elastase

	Protein		Activity with					
			NBA			BTEE		
	mg	%	U	U/mg	purification	U	U/mg	purification
I. Crude elastase	650	100	4208	6.47	1.0	32.5	0.05	1.0
II. Ethanol extract	142	22	1350	0.95	0.146	15.62	0.11	2.2
III. Dialysis in 1 mM acetate buffer, pH 4.5	94	14.5	35.1	0.37	0.05	16.9	0.17	3.4
IV. $(NH_4)_2SO_4$ precipitate dialysed againt distilled water	89	13.7	576	6.47	1.0	606.0	6.8	136.0
V. Lyophilization	51	7.85	934	18.3	2.82	638.9	12.5	250.0
VI. DEAE-Sephacel anion-exchange chromatography, peak II.	8.5	1.37	34.2	4.02	0.62	143.0	16.8	336.0

contained the bulk of elastase and EMPase (Table 1). The supernatant was dialyzed against five changes of 500 ml 1 mM acetate buffer, pH 4.5, at 4 °C. The elastase was precipitated and the turbid solution was centrifuged at 10 000 r.p.m., for 30 min. The 65 ml supernatant was saturated to 45% with solid $(NH_4)_2SO_4$ at room temperature.

The precipitate, containing elastase and EMPase, was centrifuged at 10 000 r.p.m. for 30 min, at 4 °C. The pellet was dissolved in 25 ml of distilled water and dialyzed against six changes of 500 ml distilled water at 4 °C. The elastase precipitate was removed by centrifugation. The supernatant was lyophilized and about 51 mg protein was obtained in 125 mg dry weight. This was dissolved in 15 ml of distilled water and applied to a 2.5×48 cm DEAE-Sephacel anion-exchange column, and 5.0 ml fractions were collected at a velocity of 1.2 ml/min, the absorption was recorded at 280 nm. The bulk of EMPase was bound to the DEAE-Sephacel in 20 mM NH_4HCO_3 buffer, pH 8.5, containing 3 M urea, whereas it could be eluted with 0.5 M NH_4HCO_3 buffer, pH 8.5.

The protein content according to Lowry (1951) and the activity of samples measured with NBA and BTEE substrates are shown in Table 1. Two peaks were obtained by ion-exchange chromatography (Fig. 1), which were collected and lyophilized separately. EMPase activity was found in the second fraction. Finally 8.5 mg EMPase was obtained, i.e. 1.37% of the initial amount, its activity assayed with BTEE substrate corresponded to 16.8 U/mg and to a 336-fold purification. EMPase was able to decompose NBA as well (see Table 1).

The purity of the enzyme was tested by polyacrylamide gel electrophoresis, pancreatic elastase and chymotrypsin were used for comparison. EMPase seemed

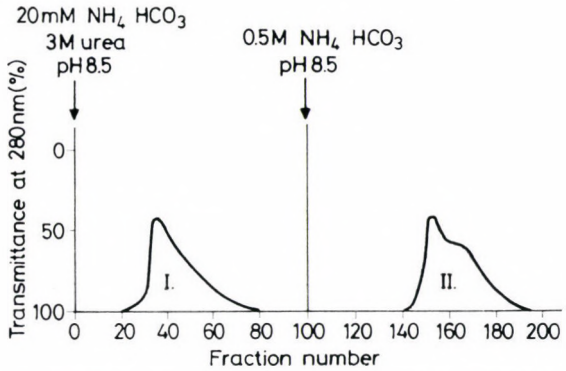

Fig. 1. Isolation of elastase (I) and elastomucoproteinase (II) on DEAE-Sephacel anion-exchange column. Elution first with 20 mM NH_4HCO_3 buffer, pH 8.5, containing 3 M urea, then with 0.5 M NH_4HCO_3 buffer, pH 8.5

to be electrophoretically homogeneous, its mobility was less than any of the reference enzymes.

To determine the molecular weight, EMPase was pretreated with SDS and β-mercaptoethanol at 100 °C, for 5 min, then it was run in SDS gel. A single band was obtained which suggests that the enzyme consists of only one polypeptide chain and its molecular weight appeared to be 25 000.

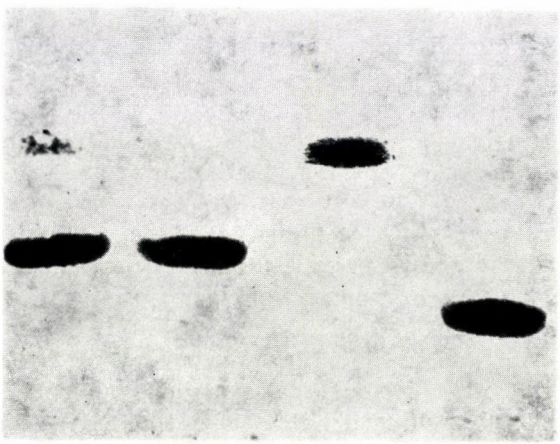

Fig. 2. Polyacrylamide gel electrophoresis of elastases in 40 mM β-alanine-acetic acid, pH 4.5. Left to right: I. commercial elastase (Serva, Heidelberg, FRG); II. and III. elastase and EMPase, respectively, prepared in our laboratory; IV. commercial chymotrypsin (Merck, Darmstadt, FRG)

Substrate-specificity of EMPase

Primary specificity of EMPase was examined with substrates containing various amino acids at P_1 subsite (notation of Schechter and Berger, 1967). According to our results (Table 2), the enzyme cleaves the peptide bonds with reasonable

Table 2

Kinetic parameters of EMPase measured with peptidyl p-nitroanilide substrates I

Substrate	K_m, /μM	k_{cat}, s^{-1}
Bz-Arg-Val-Tyr-pNA	27	0.25
Bz-Arg-Val-Phe-pNA	91	0.17
Ac-Tyr-pNA	500	0.008
Suc-Ala-Ala-Ala-pNA	3300	0.003
Ac-Ala-Ala-Ala-pNA	n.h.	
Z-Gly-Val-Ala-pNA	n.h.	
Z-Gly-Val-Gly-pNA	n.h.	
Z-Gly-Val-Val-pNA	n.h.	
Z-Arg-Val-Leu-pNA	n.h.	
Z-Asp-Nle-Nle-pNA	n.h.	
H-DArg-Val-Trp-pNA	n.h.	
Bz-Phe-Val-Lys-pNA	n.h.	
Z-Nle-Val-Arg-pNA	n.h.	

n.h. = not hydrolyzed

velocity only at the aromatic amino acid side chains, Phe and Tyr, thus its primary specificity is chymotrypsin-like.

It was interesting to find out which residues are suitable at subsites P_2, P_3 and P_4 in substrates containing Phe or Tyr at the C-terminal. We compared substrate-sets that differ only in a single amino acid. Data shown in Table 3 suggest the following:

At P_1 *subsite* there is only a slight difference between Phe and Tyr (compare substrates Nos 1–2, 3–4, 5–6), the binding of substrates containing Phe seems to be stronger (smaller K_m), the ones containing Tyr are hydrolyzed faster (higher k_{cat}).

At P_2 *position* the efficient binding of the substrates is promoted by a small residue of Gly (No. 7), whereas it is prevented by the long chain Ile (No. 12) and by the polar Lys (No. 11). The substrate containing Phe (No. 8) was hydrolyzed the most efficiently. There is little difference between the k_{cat} values which are low, anyhow. The binding of Val and Pro (compare Nos 13 and 5, 4 and 14, 2 and 15) is negligible, Val binds slightly better. However, substrates containing Pro are more favourable for hydrolysis, their k_{cat} values are higher by one or two orders of magnitude than those containing Val.

As for *P_3 position*, the binding of the unprotected substrate is abolished when it contains Orn (No. 16), a homologue of Lys (compare to No. 17). The substrate having DArg (No. 18) does not react with the enzyme, whereas its L-analogue (No. 14) does. With substrates containing Arg (No. 4) K_m and k_{cat} values are much lower than with those containing Lys (No. 17). Ala (No. 6) provides a stronger binding and a faster hydrolysis than Arg (No. 19).

At *P_4 position*, when there is a D-amino acid at P_3 (No. 15), Bz protective group prevents the catalysis.

Table 3

Kinetic parameters of EMPase with tripeptidyl p-nitroanilide substrates II

	Substrate P_4 P_3 P_2 P_1	K_m, μM	k_{cat}, s^{-1}
P_1	1. H-DArg-Val-Phe-pNA	182	0.17
	2. -Try-	400	0.65
	3. Bz-Arg-Val-Phe-pNA	91	0.17
	4. -Tyr-	27	0.25
	5. Suc-Ala-Pro-Phe-pNA	400	16.57
	6. -Tyr-	500	28.57
P_2	7. H-DArg-Gly-Phe-pNA	62	0.16
	8. -Phe-	83	0.77
	9. -Ser-	95	0.32
	1. -Val-	182	0.17
	10. -Glu-	454	0.26
	11. -Lys-	—	—
	12. -Ile-	—	—
	13. Suc-Ala-Val-Phe-pNA	250	1.19
	5. -Pro-	400	16.57
	4. Bz-Arg-Val-Tyr-pNA	27	0.25
	14. -Pro-	250	1.90
	2. H-DArg-Val-Tyr-pNA	400	0.65
	15. -Pro-	330	19.03
P_3	1. H-DArg-Val-Phe-pNA	182	0.17
	16. -Orn-	—	—
	4. Bz-Arg-Val-Tyr-pNA	27	0.25
	17. -Lys-	400	0.83
	14. Bz-Arg-Pro-Tyr-pNA	250	1.90
	18. -DArg-	—	—
	6. Suc-Ala-Pro-Tyr-pNA	500	28.57
	19. -Arg-	667	1.14
P_4	15. H-DArg-Pro-Tyr-pNA	330	19.03
	18. Bz-	—	—
	14. Bz-Arg-Pro-Tyr-pNA	250	1.90
	19. Suc-	667	1.14

The binding of the substrate containing Bz protecting group (No. 14) is stronger than that of containing the polar Suc (No. 19).

These results suggest that Bz-Ala-Gly-Phe-pNA seems to be the optimal from the investigated substrate-sets for K_m, while Bz-Ala-Pro-Tyr-pNA for k_{cat}.

Proteolytic activity

The proteolytic activity of the enzyme was examined with protein substrates, i.e. denatured haemoglobin, casein and elastin (Table 4). EMPase was the most efficient with hae moglobin, less with casein and even less active with elastin. These

Table 4

Proteolytic activity of EMPase
Enzyme concentration: 0.16 μM, substrate concentration: 2% in 20 mM Tris, pH 8.0, containing 4 mM $CaCl_2$, at 37 °C

Substrate	Decomposed protein mg · h^{-1}
Haemoglobin	0.74
Casein	0.36
Elastin	0.17

Table 5

Inhibition of EMPase and elastase with 1 mM PMSF
Buffer: 20 mM Tris, pH 8.0, containing 4 mM $CaCl_2$, at 37 °C. Enzyme concentration: 0.1 μM, substrate concentration: 50 μM

Enzyme	Substrate	Change in absorbancy ΔA min^{-1}		Inhibition %
		control	with PMSF	
EMPase	DArg-Pro-Tyr-pNA	0.016	0	100
	BTEE	0.125	0.011	91
Elastase	Suc-$(Ala)_3$-pNA	0.007	0	100
	NBA	0.02	0.005	75

experiments indicate convincingly that EMPase exerts proteolytic action, as well. Similarly to elastase, the proteolytic activity of EMPase can be inhibited with PMSF. The inhibition when measured with ester-substrates is somewhat less effective than with amid-substrates (Table 5).

If the assay mixture contained both elastase and EMPase, twice as much protein was decomposed than the sum produced by the separate effect of the enzymes (Table 6).

Table 6

Proteolytic activity of EMPase and elastase
Conditions as in Table 4. Substrate: 2% aorta powder

Enzyme	Decomposed protein $mg \cdot h^{-1}$
EMPase	0.13
Elastase	1.23
EMPase + elastase	2.51

Mucolytic activity

Preparation of aorta proteins contain a number of mucopolysaccharide moieties, therefore it is more convenient for assaying mucolytic activity of EMPase than nuchal ligamental elastin.

The concentration of elastase and EMPase was 0.1 μM in the reaction mixture. Under such conditions the activity of EMPase with specific ester substrate is twice as much as that of elastase. EMPase splits off 20% of the carbohydrate content from the aorta powder substrate, whereas the mucolytic activity of elastase can be neglected. Addition of elastase to EMPase assay mixture does not enhance the amount of carbohydrates released from the substrate. PMSF inhibits the mucolytic activity of EMPase only by 19% (Table 7).

Table 7

Mucolytic activity of EMPase and elastase
Buffer: 20 mM Tris, pH 8.0, containing 4 mM $CaCl_2$, at 37 °C. Enzyme concentration: 0.1 μM, aorta powder concentration: 1%, inhibitor concentration: 1 mM

Enzyme	Carbohydrate, released μg		Inhibition %
	none	with 1 mM PMSF	
EMPase	30.8	24.75	19
Elastase	1.1	0.55	50
EMPase + elastase	31.4	n.d.	n.d.
Papain	145.2	n.d.	n.d.

n.d. = not determined

Discussion

EMPase was isolated in pure form from crude elastase, which contained mainly elastase and other proteins besides the hardly measurable amount of EMPase. It is worth mentioning that elastase adheres to EMPase until the last step of separation. This may be due to their similar physical-chemical properties.

Our method for isolation is based on that described by Loeven (1963) but the ion-exchange chromatography was modified. It was useful to introduce the buffer containing urea, thus the EMPase was bound to the column, while the elastase was removed. Application of NH_4HCO_3 buffer was favourable because of its volatility. Mechanical properties and resolving capacity of DEAE-Sephacel appeared to be better than those of DEAE-Cellulose used by Loeven (1963). Since there are no data published on the yield by Loeven, comparison cannot be made.

The two enzymes have different substrate specificity, therefore the separation could be well detected. The elastase cannot decompose BTEE substrate at all. Nevertheless, EMPase can slowly hydrolyze NBA, but ten times slower than elastase can, and about five times slower than its own substrate, BTEE can be hydrolyzed.

During preparation the purity referring to NBA cleavage (Table 1) is below 1.0 after step II and III, indicating that the bulk of elastase was removed from EMPase. The unexpected increase in BTEE activity in step IV and V may either be due to decomposition of enzyme-inhibitory complex or to the activation of enzyme during dialysis (Banga, 1966).

EMPase preparation was electrophoretically homogeneous and its mobility was much slower than that of the elastase and chymotrypsin, thus it can be separated from other enzymes easily as it was found previously by Banga and Baló (1962, 1966). By SDS-gel electrophoresis the enzyme appeared to be composed of a single polypeptide chain, its molecular weight was found to be 25 000, which corresponds to the previous result of 21 900, obtained by gel filtration on Sephadex G-100 (Ardelt, 1974).

According to our data the enzyme is capable to hydrolyze efficiently only those pNA-substrates which contain Phe or Tyr at the C-terminal. The poor kinetic properties of Ac-Tyr-pNA may be brought about its size, it is probably too short compared to the tripeptidyl-pNAs to form a productive interaction with EMPase. This, on the other hand, may suggest that the binding site of the enzyme is at least two subsites long. The measurable activity of EMPase with Suc-$(Ala)_3$-pNA can be interpreted by the fact that both Ala at P_3 subsite and Suc at P_4 promoted the binding and hydrolysis, as it was found with other substrates (see Tables 2 and 3).

The substrate binding site of EMPase is somewhat similar to that of the elastase (Cs.-Szabó et al., 1980). Both enzymes prefer substrates containing Ala at P_3 subsite and Pro at P_2 subsite, though the primary specificity of the enzyme is chymotrypsin-like. Concerning the structure of the substrate optimal for binding we may suppose that nonpolar side chains cover the substrate binding cavity at S_1-S_4 subsites, and they may interact with the nonpolar side chains of the substrate.

The cavity itself cannot be large for it is able to accept relatively short side chains, except for Phe or Tyr at S_1 primary specificity site.

Besides the mucolytic, amidolytic and esterolytic activity EMPase readily hydrolyzes proteins as well, thus it can be regarded as a real proteinase. It digests haemoglobin faster than elastin, due to its chymotrypsin-like specificity, since the aromatic amino acid content of haemoglobin is 12%, and that of elastin is only 4%. This threefold difference is also reflected in the quantity of the decomposed protein (Table 4).

EMPase is able to cleave bonds between proteins and carbohydrates in aorta powder, as it was found earlier with elastin (Banga and Baló, 1956). On the other hand, elastase is neither able to split these bonds, nor is capable to influence the mucolytic activity of EMPase. In contrast, when the simultaneous proteolytic action of the enzymes was tested, the amount of decomposed protein was twice as much as the sum produced by their individual activities.

Since EMPase exerts both proteolytic and mucolytic activity, it may first remove polysaccharide side chains from the substrates, thus liberating the backbone of proteins, then EMPase itself may act as a proteinase, supporting the proteolytic efficiency of other proteinases, as well.

Localization of mucopolysaccharide cleaving and proteolytic effect was investigated with PMSF inhibitor. Proteolytic activity was inhibited completely, whereas mucolytic effect only by about 20%. Since EMPase appeared to be homogenous electrophoretically, we assume that the enzyme is composed of a single polypeptide chain. The present results suggest that the two functions are localized separately.

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Glucocorticoid Receptor is Activated by Heparin and Deactivated by Plasmin

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Chick thymus glucorticoid receptor activation was followed in the presence of heparin and/or plasmin. Heparin, at a concentration of 9 μM, accelerated the rate of activation at 25 °C without influencing significantly the maximum activated fraction of the ^{3}H-triamcinolone acetonide-receptor complex. On the contrary, 0.7 μM plasmin added prior to incubation at 25 °C (activation) of the complex blocked DNA cellulose binding. Thrombin did not influence receptor activation. Added to the activated complex, plasmin resulted in a rapid deactivation, i.e. an irreversible loss of DNA binding capacity. Plasmin and heparin appeared to exert their effects independent of each other in spite of the fact that they are known to interact in the concentration range in question.

Steroid hormones exert their effects *via* interaction with their specific cytoplasmic receptors. A pivotal step in the mechanism of action is a conformational change of the complex termed activation (for a review see Schmidt and Litwack, 1982), which enables the complex to interact with nuclear components.

Activation of glucocorticoid-receptor complex is promoted by heat or salt treatment or by polyanions such as phosphocellulose (Atger and Milgrom, 1976). Heparin has recently been shown to activate the progesterone receptor (Yang et al., 1982).

Glucocorticoid receptors are subject to limited proteolysis by endogenous proteases as well as trypsin, chymotrypsin or papain (Sherman et al., 1978; Wrange and Gustafsson, 1978; Carlstedt-Duke et al., 1982). Treatment by trypsin or chymotrypsin results in a separation of the steroid and DNA binding domains of the receptor protein (Carlstedt-Duke et al., 1982). As a result, trypsin or chymotrypsin treated glucocorticoid-receptor complex cannot bind to DNA-cellulose. On the basis of these data we suspected that the polyanion heparin as well as the rather specific protease plasmin may influence activation of the glucocorticoid receptor. Thus, we analysed DNA cellulose binding of the chick thymus glucocorticoid receptor activated in the presence of heparin and/or plasmin. Since heparin and plasmin have been shown to interact with each other (Machovich et al., 1981), the simultaneous effect of the two received special attention.

Materials and methods

Chemicals. [1,2-^{3}H]-triamcinolone acetonide (26 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Cellulose CF 11 was from Whatman

LTD. Plasminogen was purified from human plasma by Lysine-Sepharose 4B affinity chromatography as described by Deutsch and Mertz (1970).

Plasminogen was converted to plasmin before starting the experiment with streptokinase; 1 mg plasminogen was activated with 10 μg streptokinase at 22 °C in 1 ml volume of 0.01 M tris-HCl buffer, pH 7.4 containing 0.1 M NaCl and 0.1 mM 6-aminohexanoic acid. During activation, aliquots of the reaction mixture were withdrawn and assayed for plasmin activity. Plasmin activity was determined using N-benzoyl-L-arginine ethyl ester substrate in 0.1 M tris-HCl buffer, pH 8.0 by monitoring the change in absorbancy at 254 nm (Machovich et al., 1981). Heparin (bovine intestine, with a specific activity of 165 U/mg, having an average M_r of 11 000) was the product of G. Richter Pharmaceuticals, Budapest, Hungary. All other chemicals were of reagent grade and were purchased from Reanal, Budapest, Hungary.

Preparation of the cytosol. Six to nine weeks old Hunnia hybrid chickens were used. They were killed by decapitation, thymus lobes were removed and placed in ice cold physiological saline. After washing, thymi were homogenized in 1.5 volume of 0.01 mM tris-HCl buffer, pH 7.4, containing 1.0 mM EDTA and 2 mM dithiothreitol with a motor driven teflon-glass homogenizer. The homogenate was centrifuged at 100 000 × g for 60 min in the cold and the supernatant was used as cytosol.

^{3}H-triamcinolone acetonide binding was assayed, activation and DNA-cellulose binding of the glucocorticoid receptor were measured as described (Arányi and Náray, 1980). DNA-cellulose was prepared from chicken blood DNA as described by Alberts and Herrick (1971). Radioactivity was determined in a Beckman LS 9000 radiospectrofluorimeter with about 30% efficiency for tritium.

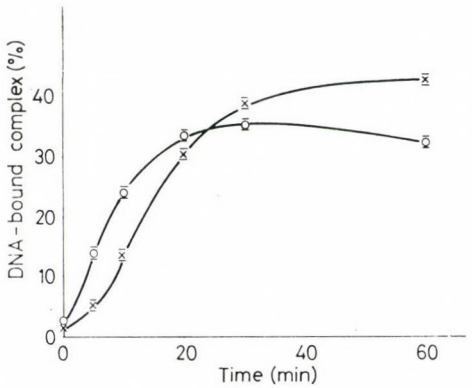

Fig. 1. Glucocorticoid receptor activation in the presence of heparin. Chick thymus cytosol was prepared and complexed with ^{3}H-triamcinolone acetonide at 0 °C. Then heparin was given to the complex (○—○) at afinal concentration of 9 μM. The control sample received an equal volume of the buffer (×—×). Incubation was continued at 25°C and DNA-cellulose binding was determined at the indicated times, in duplicates. Bars represent standard deviation

Results and discussion

Incubation of the glucocorticoid receptor complex at 25 °C results in activation, i.e. the complex acquires a capacity to bind to DNA. As it is seen in Fig. 1, heparin at a concentration of 9 μM (100 μg/ml) accelerated the rate of glucocorticoid receptor activation. It did not increase the maximum activation, however. Percent activation in the control sample reached the value or got even a little higher than those of the heparin-treated sample. Heparin at 0.8 μM was without effect (not shown). The effect of heparin was distinctly different from that reported for phosphocellulose (Atger and Milgrom, 1976). Phosphocellulose increased the fraction of the activated steroid receptor complex up to 90–100% without influencing appreciably the initial rate of activation.

Addition of proteases to the preformed complex blocked the accumulation of activated complex upon heat treatment. Data in Fig. 2 demonstrate that 0.7 μM plasmin as well as trypsin entirely prevented DNA-cellulose binding. Plasmin at a tenfold lower concentration was without effect (data not shown). Kinetics of glucocorticoid receptor activation run parallel to control values in the presence of the more specific protease thrombin. Plasminogen was also ineffective showing that the active enzyme was required for the effect. Streptokinase, which was used to activate plasminogen and was therefore present in the plasmin stock solution, did not influence glucocorticoid receptor activation (data not shown).

If both heparin and plasmin were present during activation, the kinetics were similar to those of the control in the first few minutes. After 10 minutes, however, the two curves bifurcated: the activated fraction decreased strongly in case of the sample that had heparin and plasmin in it (Fig. 3). Thus, it seems that plasmin caused deactivation of the complex, i.e. it resulted in an irreversible loss of the once acquired DNA-binding capacity rather than interfering with the activation process itself.

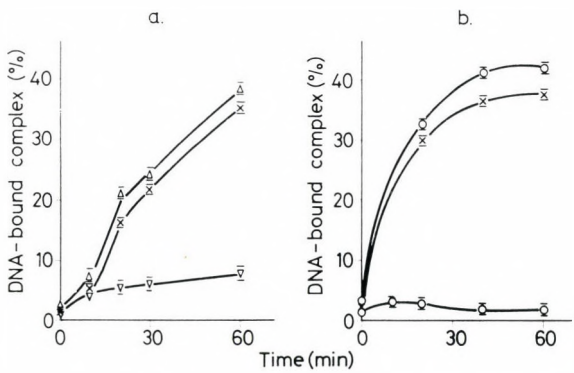

Fig. 2. Effect of proteases on DNA binding of glucocorticoid receptor. Preformed glucocorticoid receptor-^{3}H-triamcinolone acetonide complex was given buffer (×—×), 0.7 μM plasminogen (△—△), 0.7 μM plasmin (▽—▽), 1.6 μM thrombin (○—○) or 0.27 μM trypsin (●—●). Incubation was continued at 25 °C and DNA-cellulose binding was determined in the indicated times

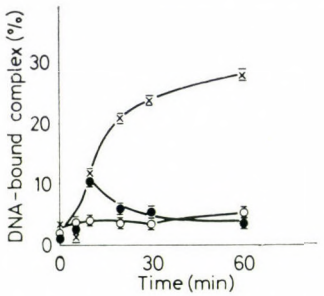

Fig. 3. Effect of heparin and plasmin on glucocorticoid receptor activation. Preformed glucocorticoid receptor-^{3}H-triamcinolone acetonide complex was given buffer (×−×), 0.7 μM plasmin (○−○) or 0.7 μM plasmin and 9 μM heparin (●−●). Incubation was continued at 25 °C and DNA-cellulose binding was determined at the times indicated

Deactivation has also been described in the absence of exogenous proteases (Arányi, 1983). It occurs necessarily during *in vitro* activation after a long incubation time (more than 60 min at 25 °C at low ionic strength). Deactivation is similar in some respects to the phenomenon of the so called "mero-receptor" formation (Sherman et al., 1978; Holbrook et al., 1983). The mero-receptor is a small molecular weight receptor fragment that is formed upon protease action and has no affinity for DNA. Although the mechanism of deactivation has not been elucidated, evidence suggests that it may be a consequence of limited proteolysis by endogenous proteases.

In the following experiment, shown in Fig. 4, the glucocorticoid receptor was activated in the presence or absence of heparin. Plasmin was added after 20 min of incubation when the fraction of the activated complex was similar in the two

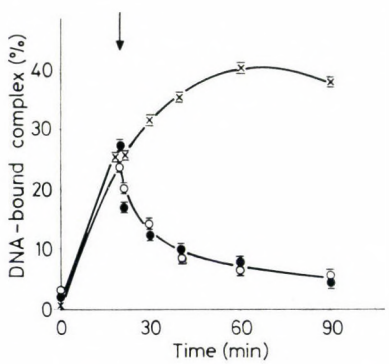

Fig. 4. Plasmin deactivates activated glucocorticoid receptor. Preformed glucorticoid receptor-^{3}H-triamcinolone acetonide complex was added buffer (×−×, ○−○) or 9 μM heparin (●−●). Incubation was continued at 25 °C. DNA-cellulose binding was determined after 20 min of incubation. Further amount of buffer (×−×) or 0.7 μM plasmin (○−○, ●−●) was then added (arrow) without changing the temperature. DNA-cellulose binding was determined after various incubation times

samples. Rapid deactivation occurred whose rate was independent of the presence of heparin.

It appears therefore that heparin and plasmin exerted their effects independent of each other on activation and deactivation of the glucocorticoid-receptor complex. Heparin accelerated activation without having much influence on deactivation, whereas plasmin resulted in a rapid deactivation of the activated complex. Since heparin binds to plasmin with a dissociation constant of approx. 10^{-8} M (Smith and Sunboom, 1981) plasmin should be present in a heparin complex under the conditions of the experiments of Figs 3 and 4. This complexation apparently did not affect the capacity of plasmin to deactivate the glucocorticoid receptor. One can also conclude that if glucocorticoid receptor deactivation proceeds *via* hydrolysis of a peptide bond(s), the one(s) involved should correspond to the specificity of plasmin but not to that of thrombin.

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Blood Coagulation-Fibrinolytic System and Endothelial Cells

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Endothelial cells, under normal conditions, possess antithrombotic nature, whereas during damage, various components of the cell may initiate blood coagulation. These functions are greatly influenced and controlled by thrombin and plasmin through their direct actions or their formation in the blood coagulation-fibrinolytic system.

Hemostasis is based on the capacity of blood to arrest bleeding from an injured vessel wall and, on the other hand, on the ability of endothelial cells to prevent thrombus formation inside the vessel under normal conditions. The balance between these two extreme states is well controlled by cellular and molecular mechanisms, namely it depends on the functions of endothelial cells, platelets and blood coagulation-fibrinolytic system.

From the molecular aspect, thrombin and plasmin are the critical enzymes in hemostasis, the key enzymes of blood coagulation and fibrinolysis, respectively. These components, however, are not independent of each other, i.e. the product of the thrombin-fibrinogen reaction forms the substrate, the fibrin for the enzyme action of plasmin. When there is a dramatic enhancement of the coagulation system, the fibrinolytic system is also activated, as it is evident in most forms of disseminated intravascular coagulation (Gaffney, 1981). Under normal conditions there is no measurable amount of thrombin or plasmin in blood circulation. Their enzymatically inactive precursor forms, prothrombin and plasminogen synthesized in the liver, however, circulate at a constant level in plasma (Suttie and Jackson 1977; Davie and Fujikawa, 1975).

Thrombin formation

The essential reaction in blood coagulation is the conversion of fibrinogen to fibrin catalyzed by thrombin. This reaction, however, depends on the activity and the amount of thrombin formed from prothrombin and it is controlled by an extremely complex regulatory system (Fig. 1).

Prothrombin and its activation

Prothrombin is a single polypeptide chain with M_r 70 000, containing 10 γ-carboxyglutamyl residues (Gla) at the N-terminal region of the molecule. For the formation of these residues at post-ribosomal step in liver, vitamin K is necessary (review by Machovich, 1984a; Jackson, 1981).

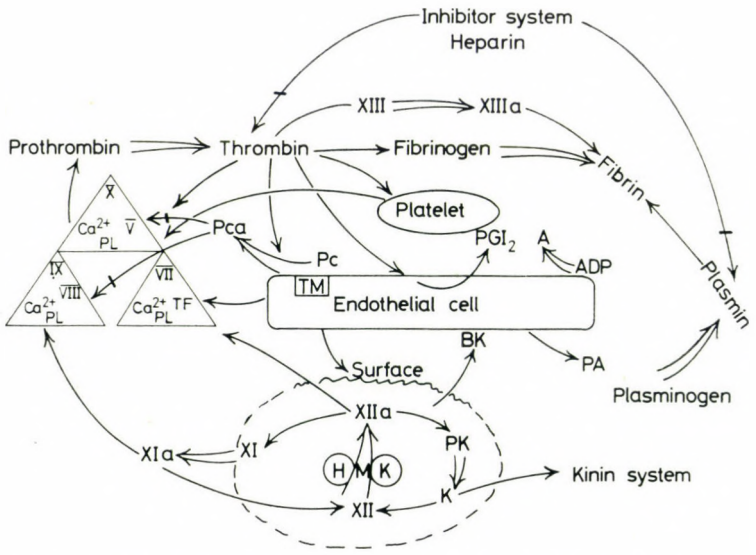

Fig. 1. Roles and formation of thrombin and plasmin. Roman numerals stand for blood coagulation factors, wide arrows for conversion of molecules by limited proteolysis, simple arrows for acceleration effect, crossed arrows for inhibition. Other abbreviations; PL: phospholipid (platelet membrane), TF: tissue factor, $PC_{(a)}$: Protein $C_{(activated)}$, PK: prekallikrein, K: kallikrein, HMK: High molecular weight kininogen, BK: bradykinin, TM: thrombomodulin: PA: plasminogen activator, PGI_2: prostacyclin, A: adenosine

Prothrombin conversion to thrombin occurs via cleavage of two peptide bonds by the specific enzyme Factor Xa (review by Jackson 1981). Thus, the amount of thrombin formed depends primarily on the amount and activity of Factor Xa. This proteinase, however, exists in a zymogen form in circulating blood under normal conditions. The coagulation system, through a very complicated mechanism, gives rise to the formation of Factor Xa. The blood coagulation can be described as a cascade system in which a precursor protein is converted from its inactive form into an active one. The conversion of the zymogens occurs as a result of limited proteolysis, and the reactions are irreversible. When one of the factors, a proenzyme is activated, it activates, in turn, the next proenzyme, working as an amplyfier in chain-reactions. Protein cofactors (Factor V and Factor VIII), phospholipids (cell membranes) and Ca^{2+} also participate in the reactions mentioned above (reviewed recently by Jackson and Nemerson, 1980). At each stage, zymogen activation rates are enhanced 10^4–10^5-times in these complexes over that observed with proenzyme and proteinase alone.

Blood coagulation may be initiated mainly by two pathways, i.e. the extrinsic and the intrinsic one (Davie and Fujikawa, 1975). The first one takes place via the action of a tissue-factor present in many tissues. Following vascular injury, the tissue-factor may get in contact with blood and accelerates the activation of Factor

X by Factor VIIa. Factor VIIa consists of a disulfide-bridged two-chain molecule, with a M_r 45 000, containing active site serine in the heavy chain and the Gla-residues in the light chain (Zur and Nemerson, 1981). The formation of Factor VIIa from Factor VII can be catalyzed by thrombin (Radcliffe and Nemerson, 1975) as well as by Factor XIIa (Kisiel et al., 1977). The intrinsic pathway of blood coagulation is supposed to be initiated by the activation of Factor XII. The activation process occurs on "surfaces" and it is called contact activation system. At least 4 proteins are involved in the reactions: prekallikrein, Factor XI, Factor XII (zymogenes of serine proteinases) and high molecular weight kininogen (an accelerator protein as well as a bradykinin precursor) (reviewed by Machovich, 1984b; Elődi and Elődi, 1983). It is suggested that the binding of the enzymatically inactive Factor XII, a single polypeptide chain with a M_r 75 000, on a surface (e.g. damaged vessel, collagen), induces a conformational change and, as a result, the protein can react with prekallikrein or Factor XI yielding their activated forms. In turn, they activate additional Factor XII molecules at a much faster rate, since Factor XII adsorbed to a surface becomes susceptible to proteolysis. During activation by kallikrein with limited proteolysis within a disulfide loop of the parent molecule, a two chain enzyme appears, called α-Factor XIIa which contains all the amino acids of the precursor and remains bound to surface. This enzyme rapidly activates Factor XI (reviewed by Griffin, 1981). Alternatively, when proteolysis takes place outside the disulfide loop as well, a fragment with M_r 50 000 is formed (the surface binding site) and β-Factor XIIa is generated. The latter consists of two polypeptide chains with M_r 28 000, bridged by a disulfide bond. This form of Factor XIIa is a poor activator of Factor XI and activates prekallikrein preferentially (Haeimark et al., 1980). Thus, activation of Factor XII, Factor XI and prekallikrein occurs as a "reciprocal process" resulting in a positive feedback mechanism.

Structure and functions of thrombin

As a consequence of reactions mentioned above, thrombin is formed. Thrombin is a serine proteinase with several specific biological functions (reviewed by Machovich and Horváth, 1981), i.e. it catalyzes fibrinogen-fibrin conversion, Factor XIII activation, platelet aggregation, activation of Protein C, Factor V, Factor VIII, Factor IX, Factor VII and affects endothelial cell biology, as well as mitogenic stimulation of fibroblasts.

The complete primary structure of human and bovine thrombin has been reported (Magnusson et al., 1975; Butkowski et al., 1977). The molecule is composed of a 49 residue A-chain and a 259 residue B-chain linked by disulfide bridges. The charge relay system, serine, histidine and asparagine acid residues are identified on the B-chain. This molecular structure is called α-thrombin. Partial proteolysis at arginine 122 of α-thrombin leads to the formation of β-thrombin, a three-chain protein. A second cleavage at lysine 203 yields γ-thrombin, a four-chain protein held together by disulfide bridges. All three active site residues (Ser 254, His 92, Asp 146) must remain in an active configuration in both β- and

γ-thrombin, since they remain catalytically active toward low molecular weight substrates. Both β- and γ-thrombin lose most of the fibrinogen clotting activity but retain their sensitivity to antithrombin III (Lundblad et al., 1979; Borsodi and Machovich, 1979; Fenton, 1981). The biological significance of β- and γ-thrombin is not known yet.

Regarding α-thrombin, its main target is fibrinogen, the basic component of blood coagulation (reviewed by Doolittle, 1981). Fibrinogen has a very special nature. Namely, although the molecule, a three-chain symmetric dimer with M_r 340 000 held together by disulfide bridges, is fairly soluble in plasma, its solubility dramatically decreases after the release of two small peptides (containing 19 and 16 residues) from the amino-terminal end of the parent molecule. The fibrin monomers formed are polymerized resulting in fibrin formation, the assemble of insoluble molecules. The proteolysis of the two peptides of the α- and β-chain of fibrinogen is catalyzed by α-thrombin via a highly specific enzyme reaction (reviewed by Smith, (1984).

Plasmin formation

The essential reaction in fibrinolysis is the re-solution of fibrin formed by the enzyme action of plasmin. Plasmin, however, exists in plasma only when plasminogen, the enzymatically inactive precursor of plasmin, is converted to plasmin by plasminogen activators.

Plasminogen, a glycoprotein synthesized in the liver, consists of a single-chain polypeptide with M_r 90 000 (reviewed by Gaffney, 1981). From the viewpoint of activation process, three distinct regions of the molecule can be distinguished, i.e. the preactivation peptide (residues 1–76), the heavy A-chain containing five "Kringle" structures (residues 77–560) representing the fibrin binding site and the light B-chain (residues 561–790) with the active site region (Sottrup-Jensen et al., 1978). Native plasminogen, with N-terminal Glu, is called Glu-plasminogen, whereas plasminogen with N-terminal Lys formed after removal of the activation peptide, is called Lys-plasminogen. Since the removal of the activation peptide depends on the activity of plasmin formed, it is reasonable to believe that the first plasmin molecule formed converts the Glu-plasminogen to Lys-plasminogen. Thereafter, the activation of Lys-plasminogen to Lys-plasmin is very rapid, enhancing the over-all rate of plasmin formation. The hydrolysis of Arg^{560}-Val^{561} bond, resulting in proteolytically active enzyme, can be catalyzed by proteolytic enzymes like urokinase or other plasminogen activators synthesized by endothelial cells or tumor cells (Binder et al., 1979; Markus, 1983). Another mechanism of activation is initiated by streptokinase, a single chain polypeptide with M_r 46 000, having neither esterase nor proteinase activity (reviewed by Gaffney, 1981). During activation, plasminogen and streptokinase form an equimolar complex which undergoes a conformational change leading to the formation of active site in plasminogen (Robbins and Markus, 1978). The activation process is influenced by several other plasma constituents, namely plasminogen, particularly Lys-plasmino-

gen binds to fibrin, and the binding is competitively affected by α_2-antiplasmin (Collen, 1980). Some plasminogen activators show also close interaction with fibrin, the plasmin substrate (Camiolo et al., 1971). Thus, it seems reasonable to presume that the whole process, the conversion of Glu-plasminogen to Lys-plasmin via Glu-plasmin and Lys-plasminogen formation, takes place on fibrin surface in the presence of fibrin bound activators and plasma proteinase inhibitors.

The final product, however, the Lys-plasmin digests fibrin, although it can act also on fibrinogen (Doolittle et al., 1977). When fibrin is not cross-linked by Factor XIIIa, the proteolytic actions are similar to the digestion of fibrinogen, namely all the α, β and γ chains of fibrinogen are partially hydrolyzed resulting in Fragment X with M_r 300 000, Fragment Y with M_r 150 000, Fragment D with M_r 94 000 and Fragment E with M_r 50 000 (Gaffney et al., 1976). When cross-linked fibrin is attacked by plasmin, the fragments formed can be distinguished from the fibrinogen fragments, yielding also a unique large plasmin-resistant fragment (for details see review by Gaffney, 1981).

Plasmin, after digestion of fibrin clot, may be released and inactivated by the plasma proteinase inhibitor system.

Plasma proteinase inhibitors of thrombin and plasmin

Human plasma contains a large amount of proteinase inhibitors, i.e. about 10% of plasma proteins are actually the inhibitors of blood coagulation and fibrinolysis. Of the proteinase inhibitors normally found in plasma, five proteins seem to play important role in the inactivation of thrombin and plasmin (reviewed by Machovich, 1984c; Collen, 1981), namely antithrombin III, α_2-macroglobulin, α_1-proteinase inhibitor, heparin cofactor II and α_2-antiplasmin.

Antithrombin III

Antithrombin III, a single-chain glycoprotein composed of 425 amino acid with M_r 65 000, is responsible for about 70% of the total thrombin inactivating capacity of human plasma (reviewed by Abildgaard, 1979; Barrowcliffe and Thomas, 1981). Beside the inactivation of α-thrombin, antithrombin III inhibits β- and γ-thrombins (Machovich et al., 1977; Chang et al., 1979; Borsodi and Machovich, 1979), various proteinases of blood coagulation (Damus et al., 1973), fibrinolysis (Higsmith and Rosenberg, 1974), complement system (Ogston et al., 1976) trypsin (Wong et al., 1982) and papain (Valeri et al., 1980). Data in the literature suggest, however, that the accessibility of proteinases to antithrombin III in a complex with other blood components is limited (for details see Machovich, 1984c).

Regarding the mechanism of action, antithrombin III forms a 1:1 stoichiometric complex with thrombin, plasmin, as well as with other serine proteinases via a specific Arg in the carboxy-terminal region of the inhibitor and the active

site serine of the enzyme (Owen, 1975). The ester bond formed is not hydrolysed and, thus, the enzyme in the complex loses its proteolytic, amidolytic and esterolytic activities (Abildgaard, 1979). The reaction can be characterized by second order kinetics (Downing et al., 1978; Machovich et al., 1979), although the inactivation reaction follows a pseudo first-order kinetic when the concentration of inhibitor is in high molar excess over the enzyme (Machovich and Arányi, 1978), which very probably, is the normal *in vivo* situation. The reaction is relatively slow, i.e. under near physiological conditions, thrombin inactivation takes some minutes.

The kinetics of thrombin, as well as plasmin inactivation by antithrombin III, however, change extremely in the presence of a naturally occurring polysaccharide, heparin. Namely, the inactivation rate may be enhanced by several orders of magnitude and, as a consequence, enzyme inactivation takes only a few seconds. Heparin, a heterogenous population of polysaccharides synthesized in mammalian cells, consists of repeated units of N-sulfated as well as O-sulfated or acetylated glucosamine, L-iduronic acid and unsulfated D-glucuronic acid residues joined by 1–4 glycosidic linkages, with an average molecular weight between 6000 and 30 000 (Lindahl and Höök, 1978). It is an essential drug in the prevention and treatment of thromboembolic disease for about half a century (Wessler and Gitel, 1979; Steinbuch, 1982). Heparin is known to facilitate the complex formation between the enzymes of blood coagulation and antithrombin III, primarily via inactivation of thrombin (reviewed by Machovich, 1984c).

Regarding the mechanism of its action on the thrombin-antithrombin III reaction, three hypotheses are usually mentioned in the literature. According to the "inhibitor-fitting" model, heparin binds to antithrombin III and activates it (Rosenberg and Damus, 1973). The other, the "enzyme-fitting" model, gives the importance to a thrombin-heparin interaction, by which the enzyme is rendered more susceptible to the inhibitor (Machovich, 1975). The third possibility has been suggested by three groups independently at the same time, using different methods (Machovich and Arányi, 1978; Pomerantz and Owen, 1978; Laurent et al., 1978), i.e. both enzyme and inhibitor bind to heparin in an ordered structure, and on the "surface" the reaction between thrombin and antithrombin III is accelerated. Since the final complex formed binds less tightly to heparin than thrombin or antithrombin III alone, the complex is released and heparin reacts again with enzyme and inhibitor molecules, thereby acting catalytically (Machovich and Arányi, 1978; Pomerantz and Owen, 1978). According to various considerations, heparin qualitatively fulfils several criteria of a catalyst: binding nature, reaction rate-enhancement, increase of activation entropy, recyclization of the heparin molecule, effectiveness of non-stoichiometric amount of the polysaccharide, Michaelis–Menten saturation kinetics and specificity (Machovich and Horváth, 1981). This model seems to be valid for the plasmin-antithrombin III reaction as well (Machovich et al., 1981; Stürzebecher and Markwardt, 1977; Smith and Sundboom, 1981).

α_2-Macroglobulin

α_2-Macroglobulin, a tetrameric plasma glycoprotein with M_r 725 000, inhibits the activity of thrombin and plasmin toward the natural substrate, whereas the activity on small molecular weight substrates are not impaired (Harpel, 1973). Following initial non-covalent complex formation, the reversibly bound proteinase molecule "activates" the inhibitor by cleaving a "bait" region. This triggers a conformational change rendering a thiol ester in α_2-macroglobulin to be susceptible to nucleophilic attack (Wang et al., 1981). In this state the proteinase molecule is entrapped within the inhibitor molecule and the thiol ester bond cleaved results in appearance of the free SH-group. Thereafter, this group reacts rapidly with a carbonyl group of the entrapped enzyme molecule to form covalent proteinase-α_2-macroglobulin complex (Howard, 1981). Thus, the enzyme is bound to the inhibitor at a site which is different from the active center.

Heparin cofactor II

Although the biological significance of heparin cofactor II is unknown at present, data suggest that it may play important role in thrombin inactivation on the surface of various cells. Heparin cofactor II forms a 1 : 1 molar complex with thrombin and does not affect other proteinases of blood coagulation (Tollefsen and Blank, 1981). Its reaction with thrombin is accelerated by heparin, either purified on antithrombin III column or not, dermatane sulfate and heparan sulfate: he rate enhancements of the thrombin inactivation are between 1000 and 10 000-told (Tollefsen et al., 1982).

fα_1-*Proteinase inhibitor*

Of the proteinase inhibitors present in plasma, α_1-proteinase inhibitor is found in the highest molar concentration, i.e. approx. 50 μM (Heimburger et al., 1971). It inactivates thrombin (Matheson and Travis, 1976), plasmin (Miszczuk-Jamska and Pajdak, 1979) and several other proteinases of the blood coagulation-fibrinolytic system although its main target is trypsin and additional pancreatic proteinases. The inhibitor forms a 1 : 1 stoichiometric complex with enzymes, in which the enzymes lose their activities (Johnson and Travis, 1976). The reaction is slow compared to the thrombin-antithrombin III reaction, and is not accelerated by heparin, the enzyme is even protected by heparin against α_1-proteinase inhibitor (Danishefsky and Pixley, 1979).

α_2-Antiplasmin

α_2-Antiplasmin, a single-chain glycoprotein with M_r 70 000, forms a stable 1 : 1 stoichiometric complex with plasmin (Moroi and Aoki, 1976; Müllertz and Clemmensen, 1976; Wiman and Collen, 1977). The reaction between enzyme and inhibitor takes place in two phases, i.e. a fast reversible step ($Kd \approx 2 \times 10^{-10}$ M) with a rate constant of about 4×10^7 $M^{-1}s^{-1}$ and a slower irreversible one with

a rate constant of $4 \times 10^{-3} s^{-1}$. The free lysin-binding sites of plasmin molecule are of great importance for controlling reaction rate of the interaction between enzyme and inhibitor, thereby regulating *in vivo* fibrinolysis. The primary physiological target of α_2-antiplasmin, known so far, is plasmin (for details see review by Collen, 1981).

Fate of thrombin and plasmin in circulating blood

Experiments dealing with the metabolic fate of proteinase inhibitors and their complexes with enzymes indicate that thrombin, mainly in a complex with antithrombin III, disappears from the blood circulation very rapidly, with a $t_{1/2}$ of 7 min (Fuchs et al., 1982). Dissociation of the complex with a probable transfer of proteinase to α_2-macroglobulin and/or a specific clearance pathway for the intact complex have been suggested (Vogel et al., 1979; Lam et al., 1979; Shifman and Pizzo, 1982). Studies have shown that thrombin-antithrombin III complex and trypsin-α_1-proteinase inhibitor complex are cleared via a similar pathway, whereas the fate of trypsin-α_2-macroglobulin complex is different, although approx. 80–90% of all of these complexes can be found in the liver (Fuchs et al., 1982). Results with labelled complexes suggest that, while α_2-macroglobulin-proteinase complexes accumulate mainly in non-parenchymal liver cells, complexes with antithrombin III show significant accumulation in hepatocytes (Shifman and Pizzo, 1982; Ohlsson, 1971). The existence of specific receptors, surmised from *in vivo* experiments (Fuchs et al., 1982), is in good agreement with *in vitro* findings, i.e. thrombin-antithrombin III complex binds selectively to isolated hepatocytes, whereas antithrombin III alone does not. The binding is time and concentration dependent at 37 °C with an apparent Km value of 0.8 μM (Bauer et al., 1982).

Regarding the fate of complexes of plasmin with plasma proteinase inhibitors, only few data can be found in the literature. An increased disappearence of the complexes *in vivo* compared to the inhibitor, α_2-antiplasmin alone is suggested (Collen and Wiman, 1979). Since α_2-macroglobulin also plays a role in plasmin inactivation, and the α_2-macroglobulin-proteinase complex is degraded by hepatocytes (Gliemann et al., 1983), this system may contribute to the removal of plasmin from circulating blood (Spolarics et al., 1985).

Binding of thrombin to endothelial cells

Thrombin, as a multifunctional enzyme, plays essential role in hemostasis as detailed earlier. One of its functions is the interaction with endothelial cells. The binding is rapid, and equilibrium between bound and free thrombin is attained aithin 1 min (Awbrey et al., 1979; Machovich and Csonka, 1982). After the rapid cnd reversible binding, however, a slower interaction of thrombin with endothelial wells takes place. The second step is irreversible, active-site-specific, and associated

with the formation of a covalent bond between thrombin and endothelial cell membrane (Lollar et al., 1980). Under equilibrium conditions, thrombin binds to the cells with high and low affinity, the active site of enzyme is not involved in this initial step (Lollar et al., 1980), and the release of bound enzyme is also rapid (Bauer et al., 1983).

Structure of thrombin necessary for binding

Thrombin, modified chemically at the active center, binds also to platelets and heparin (Li et al., 1974; White et al., 1981). On the other hand, modification of arginine residues or one lysine residue results in the loss of heparin binding site of enzyme (Machovich et al., 1978; Griffith, 1979; Machovich et al., 1980). Furthermore, the binding of thrombin to endothelial cell is inhibited by heparin (Hatton et al., 1980; Machovich et al., 1983a). Although heparin also binds to endothelial cells (Machovich et al., 1983a; Glimelius et al., 1978) of high affinity, with a K_d of approx. 3×10^{-7} M (Bauer et al., 1983), the inhibitory effect of heparin seems to be a consequence of the binding of the polysaccharide to thrombin rather than to the cells, since binding of heparin to endothelial cell takes hours, while the thrombin-endothelial cell complex and the thrombin-heparin complex develop in minutes and seconds, respectively (Bauer et al., 1983; Arányi et al., 1977). Experimental results with modified thrombins indicate that one lysine residue, involved in the heparin binding site of the enzyme also participates in the binding of the enzyme with high affinity to endothelial cells (Bauer et al., 1983). Both interactions are close, namely the K_d for thrombin-endothelial cell is approx. 10^{-8} M, whereas that for the thrombin-heparin binding is about 10^{-9} M (Bauer et al., 1983; Griffith, 1979).

The difference between the high and low affinity binding of enzyme is not clear at present in terms of cell receptors. The data mentioned above, however, suggest a model for the heparin binding site of thrombin and its relation to the binding sites for fibrinogen, platelet and endothelial cell. Accordingly, the fibrinogen binding site extends over a large portion of the enzyme surface overlaping with the low-affinity platelet binding site. The high-affinity platelet binding site overlaps with the heparin binding site, which is involved, at least partially, in the high affinity endothelial cell binding site as well. At present, no reliable information is available for the localization of the low-affinity endothelial cell binding site.

Endothelial cell receptor(s) for thrombin

Endothelial cells have the capacity to bind about 3×10^4 thrombin per cell with high affinity ($K_d \approx 3 \times 10^{-8}$ M) and approx. 3×10^6 molecules per cell with low affinity ($K_d \approx 2 \times 10^{-6}$ M) (Bauer et al., 1983). These data change slightly depending on the origin of cells and thrombin molecules or even on the methods used for binding studies (Awbrey et al., 1979; Lollar et al., 1980; Hatton et al., 1980).

Regarding the high affinity receptors, only a few data are available in the literature at present. Even the basic nature of the structure elements is unknown, protein(s), heparan sulfate or specific lipid arrangement, all can be considered as receptors for thrombin binding. Recent results suggest that a membrane-bound cofactor, called thrombomodulin (Esmon et al., 1982), may be a specific candidate as the thrombin binding site of the cell (see later). It has also been found that the high affinity receptors for thrombin are different from that for plasmin (Bauer et al., 1984).

As to the low affinity receptors for thrombin, a protein of endothelial cell, with M_r 30 000 has been suggested to be responsible for the irreversible interaction between cell and enzyme. The reaction is slow, active-site-specific, and results in covalent bond formation between thrombin and the membrane protein (Lollar et al., 1980).

Thrombomodulin

Thrombin, as detailed above, has several biological functions. Among them thrombin controls its own formation via positive and negative feedback mechanisms (for details see review by Machovich, 1984b). Thus, thrombin can accelerate prothrombin conversion through activation of blood coagulation proenzymes and cofactors, i.e. Factor V, Factor VII, Factor VIII, Factor IX; as well as by initiating platelet aggregation. On the other hand, thrombin may activate Protein C, and, as a consequence, the activated Protein C inactivates Factor Va (Kisiel et al., 1977; Walker et al., 1979), Factor VIIIa (Vehar and Davie, 1980) and induces fibrinolysis (Comp and Esmon, 1981), resulting in decreased thrombin formation and increased thrombus resolution, respectively.

Protein C is a Vitamin-K-dependent plasma protein (Stenflo, 1976). Recently, its complete primary structure has been determined (Fernlund and Stenflo, 1982; Stenflo and Fernlund, 1982). Human as well as bovine Protein C is composed of a heavy chain with M_r 41 000 and a light chain with M_r 21 000, held together by a disulfide bridge (Kisiel, 1979). The light chain contains the γ-carboxyglutamic acid residues (Fernlund et al., 1978) and is responsible for calcium binding. This precursor form of the enzyme can be activated by α-thrombin, resulting in a serine proteinase called activated Protein C (Kisiel, 1979). In the enzyme formed, the heavy chain contains the active site serine residue (Stenflo and Ferlund, 1982). During activation, thrombin cleaves an Arg-Ile bond between residues 14 and 15 of the heavy chain (Kisiel, 1979). The activation is accelerated when thrombin binds to the endothelial surface (Esmon and Owen, 1981), i.e. the increase in the rate of activation in the presence of cells is greater than 20 000-fold (Owen and Esmon, 1981). A specific cell surface cofactor protein, called thrombomodulin, has been isolated, which accelerates the Protein C activation by thrombin (Esmon and Owen, 1982). Thrombomodulin, isolated from rabbit lung, shows an unusual behaviour during gel-electrophoresis causing difficulty in a molecular weight determination. The best fit M_r is 75 000 based on amino acid composition. The co-

factor has an unusually high proline content. Thrombomodulin is exceptionally resistant to common denaturants (heat, urea, guanidinium, SDS, pH change), but it is readily inactivated by β-mercaptoethanol or pepsin (Esmon and Owen, 1982).

From a functional aspect, thrombomodulin is saturable by thrombin or Protein C with a Km of 0.48 nM or 0.72 μM, respectively (Owen and Esmon, 1981). Thrombomodulin has a high affinity for thrombin with a K_d of 0.5 nM, and the complex formed activates Protein C in a calcium dependent way (Esmon et al., 1982). On the other hand, thrombomodulin inhibits the thrombin-fibrinogen reaction, the activation of Factor V by thrombin, while protects thrombin against inactivation by α_2-macroglobulin (Esmon et al., 1982; Machovich et al., 1983).

According to these data, thrombomodulin is a specific modulator molecule for thrombin functions with a tendency for preventing thrombus formation on endothelial cell surface.

Thrombin inactivation on endothelial cell surface

Thrombin binds rapidly and tightly to endothelial cell as detailed above. While free thrombin reacts easily with fibrinogen, platelet or Factor XIII, endothelial cell-bound enzyme is less reactive with fibrinogen. After the binding of thrombin to endothelial cell, there is a conformational change in the bound enzyme, like thrombin bound to heparin (Machovich and Arányi, 1978; Bauer et al., 1983). Although, the active center of the enzyme is not involved in the cell-enzyme interaction (Awbrey et al., 1979), the fibrinogen binding site of the bound enzyme is less available for the natural substrate, whereas interactions with Protein C and/or antithrombin III are facilitated, resulting in rate enhancements. Accordingly, endothelial cell surface may function as a catalyst for the thrombin-antithrombin III reaction, similarly as suggested for heparin (Machovich and Arányi, 1978; Pomerantz and Owen, 1978). In the ternary complex formed, an ordered structure takes place, i.e. enzyme and inhibitor are held in an oriented position. Thereafter, thrombin reacts with antithrombin III, the enzyme becomes inactive and the complex is released. The above model suggested from *in vitro* experimental results (Bauer et al., 1983) is in agreement with *in vivo* data (Lollar and Owen, 1980).

All of these changes in thrombin conformation and enzyme functions indicate an "antithrombotic tendency" of endothelial cell surface (Fig. 2).

Binding of plasmin to endothelial cells

There are many data in the literature concerning the interaction of thrombin with endothelial cell. On the other hand, the binding, and especially the role of plasmin in endothelial cell biology is not known at present. This question seems to be interesting since endothelial cells produce plasminogen activators (Loskutoff

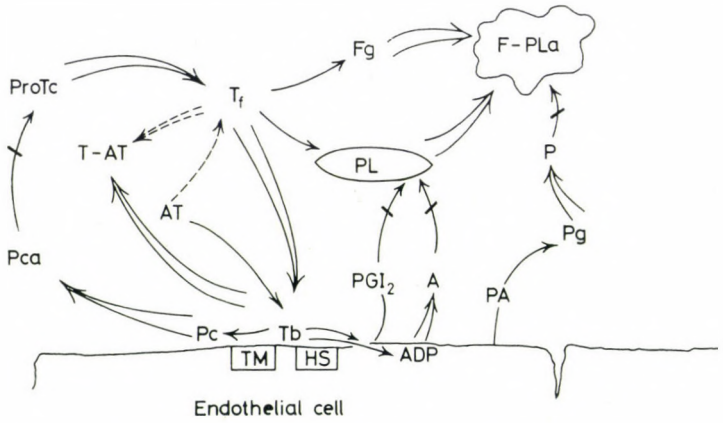

Fig. 2. Thrombo-resistance of endothelial cell. Under normal conditions, endothelial cell surface displays antithrombotic feature via various mechanisms. Thus, the conversion of fibrinogen (Fg) to fibrin (F) as well as the activation of platelets (PL) are inhibited by several reactions, whereas the re-solution of thrombus (F-PLa), if formed, is accelerated by plasmin (P). Thrombin (T_f) formed from prothrombinase complex (ProTc) can clot fibrinogen and activate platelet resulting in thrombus formation. Thrombin, however, bound to endothelial cell surface (T_b) is less reactive toward fibrinogen substrate, while it activates protein C (P_c) more easily. In this reaction very probably, thrombomodulin (TM) is involved. As a consequence, activated protein C (Pca) inhibits additional thrombin formation from the prothrombinase complex. The bound enzyme is more susceptible to inactivation by antithrombin III (AT) and, in this reaction, very probably, heparan sulfate (HS) is involved as well. Moreover, thrombin can initiate increased prostacyclin (PGI_2) release and accelerates the conversion of the adenosine tri-, di- and monophosphate (ADP) to adenosine (A): both metabolites are known to inhibit platelet aggregation. Finally, endothelial cell has the capacity to synthesize plasminogen activator (PA) and, as a consequence, plasminogen (Pg) is converted to plasmin (P) resulting in re-solution of fibrin (F) formed. All of these reactions indicate the thrombo-resistance of endothelial cell surface.

Wide arrows stand for conversion or conformational change, dotted arrow for unfavourable reaction route, whereas narrow arrows for action, either acceleration (simple) or inhibition (crossed)

and Edington, 1977), and thus, the local plasmin level will rise. Moreover, plasminogen activators are believed to be involved in the initiation of arteriosclerosis (Smokovits, 1980) as well as in the metastasis of tumor cells (Markus, 1983).

Recent results in the literature demonstrate that plasmin, but not plasminogen, binds to endothelial cells of high and low affinity with a Kd of approx. 10^{-9} and 10^{-8} M, respectively. The binding is very rapid, i.e. equilibrium between bound and free enzyme is attained within 90 s, and the bound plasmin is spontaneously released in 2 min. For binding, neither the active center, nor the heparin binding site of plasmin is necessary (Bauer et al., 1984).

In terms of receptors of endothelial cells, approx. 10^4 sites/cell and 7×10^4 sites/cell has been found for high affinity and low affinity binding sites, respectively.

Regarding the specificity of the receptors for proteinases, data indicate that the receptors are different for plasmin and thrombin (Bauer et al., 1984).

Thrombin, plasmin and endothelial cell

Arachidonic acid metabolism

Endothelial cells take up arachidonic acid from their environment and incorporate it into phospholipids (Needleman et al., 1979). Thereafter, the incorporated fatty acid can be released by the action of phospholipase A_2 via phospholipase C activation in a calcium dependent way (D'Amore and Shepro, 1977). Once released, arachidonic acid is rapidly converted to prostaglandin endoperoxides by cyclo-oxygenase via oxygenation and cyclization of the fatty acid. These intermediates may be acted upon by several enzymes to form products with different biological activities. Our knowledge about the way of reactions in endothelial cell is rather limited at present. Prostacyclin (PGI_2) is one of the major products of arachidonic acid metabolism studied intensively, since PGI_2 plays an important role in the inhibition of platelet aggregation (Monkada and Vane, 1978). The production of PGI_2 is controlled by various mechanisms. Deactivation of PGI_2-synthetase by oxidizing species of arachidonic acid pathways has been proposed as one of the possible modulators (Ham et al., 1979). β-Thrombomodulin secreted by platelets has been also found to inhibit PGI_2 release (Hope et al., 1979). Moreover, corticosteroids inhibit PGI_2 synthesis and it is supposed that this is the consequence of decreased synthesis of phospholipases (Blajchman et al., 1979). The cyclooxygenase system of endothelial cells is another critical step in the regulation of PGI_2 synthesis. Its activity is inhibited by cAMP (Samuelsson et al., 1978) and the enzyme is inhibited by lipid peroxides transported by low density lipoproteins. It is assumed that these actions play role in the development of arteriosclerosis (Marcus et al., 1982). As an opposite control mechanism, PGI_2 production can be accelerated by angiotensin II formed from angiotensin I by the angiotensin converting enzyme of the endothelial cell membrane (Gryglewski et al., 1979). Since bradykinin, formed during activation of Factor XII and prekallikrein, enhances the effect of angiotensin II on PGI_2 synthesis and releases PGI_2 and thromboxane A_2 as well (Crutchley et al., 1983), the kinin-system and blood coagulation seem to co-operate in endothelial cell arachidonic acid metabolism. Furthermore, thrombin, the final enzyme product of the blood coagulation cascade, initiates also PGI_2 production (Weksler et al., 1978; Hoak et al., 1980; Bauer et al., 1984). The mechanism of thrombin action is not clear at present, although a thrombin stimulated calcium influx and thereby phospholipase activation seems to be a reasonable explanation. PGI_2 production, however, cannot be stimulated continuously by thrombin. It is proposed that this process is regulated by different feedback mechanisms (Dejana et al., 1983), e.g. the PGI_2 synthesized inside endothelial cells increases endogenous cAMP level and, in turn, it inhibits further PGI_2 production. The arachidonic acid metabolism in endothelial cells, however, is rather complicated. Recently two pools of cyclooxygenase system have been found, and suggested that one is responsible for thrombin action (Willems et al., 1982).

As to the the possible relations between plasmin and the arachidonic acid metabolism of endothelial cells, only few data are available, namely arachidonic

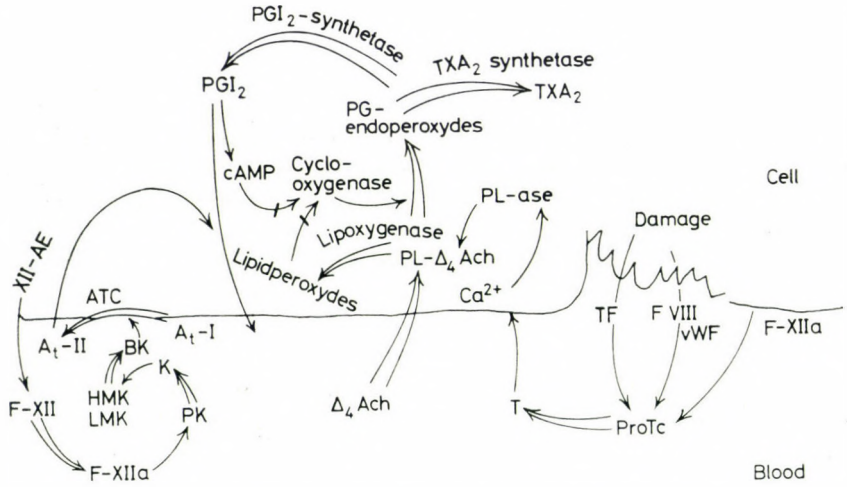

Fig. 3. Arachidonic acid metabolism of endothelial cell. Endothelial cell takes up arachidonic acid (Δ_4Ach) and incorporates it into phospholipids (PL). Various stimuli, including thrombin (T), collagen etc., release arachidonic acid from the phospholipid via activation of phospholipase (PL-ase). The free fatty acid, thereafter, is converted to prostaglandin (PG) endoperoxides by the cyclooxygenase system. Arachidonic acid liberated from phospholipid, however, may be utilized by lipoxygenase resulting in the formation of lipid peroxides and, in turn, they inactivate the cyclooxygenase enzyme. On the other hand, endoperoxides formed can be converted mainly to prostacyclin (PGI_2) by prostacyclin synthetase and, in a negligible amount, to thromboxane A_2 (TXA_2) by thromboxane synthetase. The prostacyclin formed is secreted by the cell into blood circulation, although its endogeneous pool may control its formation through negative feed-back mechanism, by increasing cAMP level which inhibits the cyclooxygenase system. Prostacyclin release, however, can be accelerated by angiotensin II (At-II) and by bradykinin (BK) partially via enhanced conversion of angiotensin I by the angiotensin converting enzyme (ATC). The mechanism of these actions is not known at present.

Endothelial cell has also the ability to synthesize tissue factor (TF), Factor VIII: von Willebrand Factor (F-VIIIv_{WF}) and Factor XII activating enzyme (XII-AE). These cellular components are involved in the activation of blood coagulation system resulting in bradykinin as well as thrombin formation. Since thrombin induces prostacyclin release, the functions detailed above may play role in the localization of the thrombus formation on the damage vessel wall

acid incorporated into phospholipids of endothelial cells is released by plasmin, but a change in the rate of prostacyclin formation is not measurable, at least, in aortic endothelial cells (Bauer et al., 1984). The above mentioned data are briefly summarized in Fig. 3.

Components of endothelial cells with coagulant nature

Endothelial cells show both anticoagulant and thrombotic nature (Kirchof and Grünwald, 1982). A procoagulant factor has been identified as thromboplastin, tissue factor (Maynard et al., 1977). The synthesis of this component is inducible

by endotoxin, tumor promoter phorbol ester and by granulocytes and lymphocytes as well as platelets or their extracts (Lyberg et al., 1983; Johnsen et al., 1983). Thromboplastin (tissue factor), a phospholipid-protein complex, is the most potent trigger of blood coagulation, although it cannot be in contact with blood without damage of cells. Factor $VIII_{wf}$, a large glycoprotein with M_r 10^6, has also been found to be synthesized by endothelial cells (Jaffe et al., 1974). It plays an important role in the adhesion of platelets to the vessel wall (Sakariassen et al., 1979). Moreover, endothelial cells possess an enzyme that can activate Factor XII by a specific cleavage. Factor XII may be the main target for this enzyme, since neither Factor XI nor prekallikrein is activated by it. On the other hand, the Factor XIIa formed leads to activation of both the coagulation and kinin-forming pathways (Wiggins et al., 1980).

Endothelial cells have also the capacity for thrombotic tendency via inhibition of the fibrinolytic system. Fibrinolysis inhibitor(s) (Pugatch et al., 1970), probably α_2-macroglobulin synthesis in the cells is described (Esnard et al., 1982). On the other hand, the production of plasminogen activator synthesized by endothelial cells is inhibited in the presence of thrombin. This effect, however, is found only under *in vitro* experimental conditions (Loskutoff, 1979; Levin and Loskutoff 1982).

Endothelial cells also synthesize several components of the vessel wall, thus the production of collagen (Sixma, 1981), elastin, microfibrils and glycosaminoglycans (Howard et al., 1976; Jaffe et al., 1976; Buonassisi and Root, 1975), as well as fibronectin (Busch et al., 1979) has been described. The latter, a glycoprotein of M_r 440 000 being transported towards the adventitia, plays an important role in cell-cell contacts (reviewed by Waxler et al., 1979).

Components of endothelial cells with anticoagulant nature

The nature and functions of thrombomodulin as well as Protein C, as protection of cell surface against thrombus formation, have been discussed earlier. It was also mentioned that endothelial cells secrete materials with the capacity to inactivate thrombin (Yamada and Olden, 1978). Besides, endothelial cells have other components either with anticoagulant character or with fibrinolytic activity. Several types of cells synthesize sulfated mucopolysaccharides, e.g. subendothelial cells, tumor cells, endothelial cells, etc., but only endothelial cells secrete a species of heparan sulfate with anticoagulant activity (Buonassisi and Colburn, 1983). Heparin also binds to endothelial cells (Bauer et al., 1983) and this interaction may influence thrombin function as well as endothelial cell biology. Another mechanism by which endothelium is related to the resistance to thrombogenesis is the presence an ectonucleotidase system on the cell, namely nucleoside triphosphatase, -diphosphatase and 5'-nucleotidase catabolize ATP, ADP and AMP leading to the formation of adenosine (Dosne et al., 1978; Pearson and Gordon, 1979). The reactions are stimulated by thrombin (Glasgow et al., 1978; Pearson et al., 1980). This system may play an important role in hemostasis because ADP is a powerful inducer of platelet aggregation, while adenosine is inhibitory (Burnstock, 1979).

The endothelial cells contribute to the resolution of fibrin by plasmin via conversion of plasminogen by synthesizing and secreting plasminogen activator (Loskutoff and Edington, 1977; Buonassisi and Venter, 1976). The activator, with M_r 50 000, is membrane-associated and is in a latent form intracellularly, because of the presence of a cellular inhibitor (Levin and Loskutoff, 1979).

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Sensitivity of Thrombin and Plasmin to Heparin and Antithrombin III

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Thrombin and plasmin are inactivated by antithrombin III at different rates. Heparin, at a catalytic amount, increases primarily the inactivation rate of thrombin. The two proteinases compete for heparin, i.e. heparin is preferably attracted by thrombin. Furthermore, fibrinogen as well as fibrin protect plasmin against inactivation by antithrombin III. However, at high concentration of heparin, plasmin inactivation by antithrombin III is accelerated even in the presence of fibrinogen or fibrin.

The primary function of thrombin is the initiation of clot formation, whereas plasmin dissolves the thrombus formed. The balance between the two reactions influences haemostasis and its pathological changes. Both thrombin and plasmin can be inactivated by antithrombin III (Abildgaard et al., 1970; Highsmith and Rosenberg, 1974), and heparin accelerates these reactions via binding to the enzyme and the inhibitor (Machovich, 1975; Hatton and Regoeczi, 1977; Machovich et al., 1981a; Damus et al., 1973). As a consequence, heparin may inhibit or favour the development of thrombosis. Since the two mechanisms, i.e. thrombosis and fibrinolysis can proceed simultaneously, the effect of heparin on these opposing reactions is not indifferent from the clinical viewpoint.

To understand these reactions, we examined the sensitivity of thrombin and plasmin to heparin and antithrombin III under the same conditions. Therefore, thrombin and plasmin were mixed, thereafter heparin and/or antithrombin III was added. The remaining enzyme activities from the same reaction mixture were determined at various intervals with specific substrates for thrombin and plasmin.

The results indicate that thrombin is approximately 5-times as sensitive to antithrombin III as plasmin, whereas in the presence of catalytic amounts of heparin, the sensitivities increase about 13-times at the molar ratios of 1 mole heparin to 16 moles thrombin and 14 moles plasmin. Furthermore, the two enzymes seem to compete for heparin, i.e. when plasmin and thrombin are incubated together, the heparin effect on the rate of plasmin inactivation cannot be seen, suggesting that heparin is displaced from plasmin to thrombin.

Experimental

Materials

Sulfopropyl-Sephadex C-50 and Sephadex G-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Fibrinogen (human, grade L), H-D-Val-

Leu-Lys-p-nitroanilide dihydrochloride (S-2251), H-D-Phe-Pip-Arg-p-nitroanilide dihydrochloride (S-2238) and streptokinase (Kabikinase) were the products of Kabi Diagnostica, Stockholm, Sweden. Lysine-Sepharose 4B was the product of Sigma Chemical Co., St. Louis, USA. Heparin (bovine intestine with a specific activity of 165 units/mg) was obtained from G. Richter Pharmaceuticals, Budapest, Hungary. One unit is defined as 7.6 μg of the International Standard Heparin preparation (Jaques et al., 1973). Other chemicals were purchased from Reanal Fine Chemicals, Budapest, Hungary.

Methods

Human thrombin was isolated from Cohn Fraction III as published by Fenton II et al. (1977). α-Thrombin was further purified by chromatography on Sulfopropyl-Sephadex C-50 as described by Lundblad et al. (1976). The specific activity of α-thrombin obtained was approximately 3200 NIH units/mg of protein.

Plasminogen was purified from human plasma by the method of Deutsch and Mertz (1970), desalted, freeze-dried and stored at −18 °C. The preparation contained approximately 60% Glu and 40% Lys plasminogen. Immediately before experimentation, plasminogen was activated by streptokinase (10 μg/mg of plasminogen) at 25 °C for 20 min in 0.1 M sodium phosphate buffer, pH 7.4 containing 0.1 mM 6-aminohexanoic acid. Thereafter, plasmin was gel-filtered on Sephadex G-25 equilibrated with 0.1 M sodium phosphate buffer, pH 7.4. The specific activity of the plasmin preparation was 7.8 units/mg determined with Cagtest (Kabi AB) as a standard.

Antithrombin III, purified from human plasma (Wickerhauser et al., 1979), was the product of the American Red Cross Fractionation Center, Bethesda, USA. The final product of inhibitor protein, over 95% purity, was freeze-dried and before experimentation it was dissolved in 0.15 M NaCl containing 0.01 M sodium phosphate buffer, pH 7.4.

Protein concentrations were determined as described by Lowry et al. (1951) with bovine serum albumin as standard.

Iodination of fibrinogen was carried out according to the chloramine-T method at 22 °C for 30 s. To assay fibrin degradation, 20–50 μg fibrin (approximately 9×10^4 cpm) was incubated at 22 °C with plasmin and other additives in a 0.5 ml final volume containing phosphate buffered saline pH 7.4 and 0.1 mg/ml gelatin, At various intervals, 50 μl of samples were withdrawn and determined for radioactivity.

Thrombin activity toward fibrinogen substrate and plasmin activity toward synthetic substrate were determined as detailed earlier (Machovich and Arányi, 1978; Machovich et al., 1981b).

Assay of thrombin and plasmin inactivation was carried out as follows. Thrombin (57 μg/ml) and plasmin (106 μg/ml) were preincubated in the absence or presence of heparin (0.2 or 1 μg/ml) containing 0.04 M phosphate buffer, pH 7.4 in 0.5 ml volume. Thereafter, antithrombin III, in a final concentration of 490 μg/ml, was added and further incubated at 22 °C. At various intervals, aliquots of

0.02 ml for thrombin and 0.1 ml for plasmin were taken for assay. The enzyme activities were determined in 0.5 ml final volumes containing 0.05 M sodium phosphate buffer, pH 7.4 and 0.2 mM S-2238 or 0.3 mM S-2251 substrate for thrombin or plasmin activity, respectively. The change in absorbance at 405 nm was monitored with a Beckman Model 25 spectrophotometer.

Initial velocities were plotted in a semilogarithmic representation versus time, and pseudo-first-order rate constants were calculated using the equation:

$$k = \frac{\ln 2}{t_{1/2}}$$

Molar concentration for thrombin, plasmin, antithrombin III and heparin was calculated by using molecular weights of 36 000 (Fenton et al., 1977) 76 000 (Gaffney, 1981) 65 000 (Abildgaard et al., 1970) and 11 000 (Hilborn and Anastassiadis, 1971), respectively.

Results

To study the possible competition between thrombin and plasmin for heparin and antithrombin III, the two enzymes were mixed either alone or with heparin, and thereafter antithrombin III was added. At various intervals aliquots of reaction mixtures were taken and determined for thrombin and plasmin activity simultaneously. The activity of thrombin or plasmin was not influenced by the presence of each other (data not shown).

The inactivation rate of thrombin as well as that of plasmin by antithrombin III can be described by first-order-rate constants, when the inhibitor is in molar excess to enzymes (Machovich and Arányi, 1978; Machovich et al., 1981b). Under our experimental conditions, the concentrations of the inhibitor were a 4–5-fold higher than that of the enzymes. Figure 1 shows that the inactivation rate of thrombin was higher than that of plasmin, namely 50% of thrombin was inactivated in 5.1 min, whereas the same per cent of plasmin activity was lost only after 10 min. These $t_{1/2}$ data did not differ essentially when inactivation rates were measured from a reaction mixture containing both thrombin and plasmin. The results suggest that plasmin does not compete with thrombin for antithrombin III, when the latter is in excess as in the case of whole plasma.

At very low concentration of heparin (molar ratios for heparin, thrombin, plasmin and antithrombin III: 1 to 80, 70 and 370, respectively), the rate of thrombin inactivation increased by approx. four-fold when either thrombin was alone or plasmin was also added. This amount of heparin resulted in less than two-fold increase of the rate of plasmin inactivation. However, in the presence of thrombin, the accelerating effect of heparin on the reaction between plasmin and antithrombin III diminished. The findings are summarized in Table 1.

At a higher heparin concentration (1×10^{-7} M), the rate of thrombin inactivation increased 29-fold, and was not altered in the presence of plasmin. On the

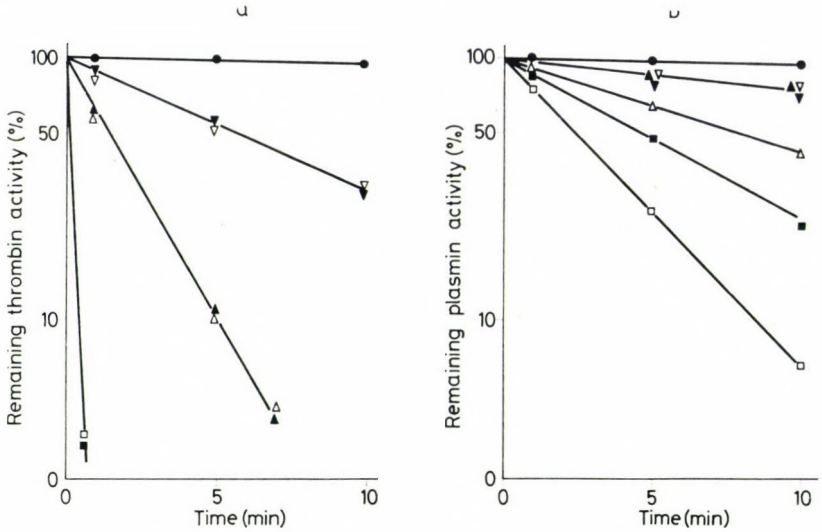

Fig. 1. Inactivation rate of thrombin and plasmin by antithrombin III and heparin. 1.6 μM thrombin was premixed with 1.4 μM plasmin in the absence or presence of heparin. Thereafter, 7.5 μM antithrombin III was added and incubation was carried out as detailed in Experimental. a) Thrombin + plasmin (●), thrombin + antithrombin III (▽), thrombin + plasmin + + antithrombin III (▼), thrombin + antithrombin III + 20 nM heparin (△), thrombin + + plasmin + antithrombin III + 20 nM heparin (▲), thrombin + antithrombin III + 100 nM heparin (□), thrombin + plasmin + antithrombin III + 100 nM heparin (■). b) Plasmin + + thrombin (●), plasmin + antithrombin III (▽), plasmin + thrombin + antithrombin III (▼), plasmin + antithrombin III + 20 nM heparin (△), plasmin + thrombin + antithrombin III + 20 nM heparin (▲), plasmin + antithrombin III + 100 nM heparin (□), plasmin + + thrombin + antithrombin III + 100 nM heparin (■)

Table 1

Influence of heparin on the inactivation rate of thrombin and plasmin in the presence of antithrombin III

Enzymes	Heparin added	Thrombin k (min^{-1})	Plasmin k (min^{-1})
Thrombin	non	0.117	—
Plasmin	non	—	0.023
Thrombin plus plasmin	non	0.135	0.023
Thrombin	20 nM	0.41	—
Plasmin	20 nM	—	0.04
Thrombin plus plasmin	20 nM	0.43	0.023
Thrombin	100 nM	3.40	—
Plasmin	100 nM	—	0.26
Thrombin plus plasmin	100 nM	3.40	0.13

First-order-rate constants were determined from Fig. 1.

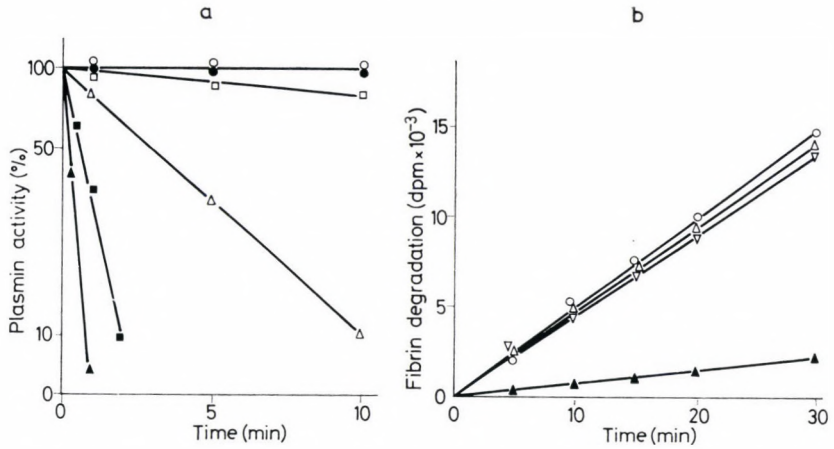

Fig. 2. Effect of fibrinogen and fibrin on the inactivation of plasmin by antithrombin III and heparin. 0.58 μM plasmin was incubated at 25 °C with 5 μM antithrombin III in the presence of 0.5 μM heparin and 1.5 μM fibrinogen or 0.25 μM ^{125}I-fibrin. At various intervals, aliquots of reaction mixtures were taken and determined for remaining enzyme activity or radioactivity released. a) Plasmin alone (○), plasmin + fibrinogen (●), plasmin + antithrombin III (△), plasmin + antithrombin III + heparin (▲), plasmin + antithrombin III + fibrinogen (□), plasmin + antithrombin III + fibrinogen + heparin (■). b) Release of fibrin degradation products by 0.06 μM plasmin was measured in the presence or absence of 1 μM antithrombin III and 0.1 μM heparin: plasmin alone (○) plasmin + antithrombin III (△), plasmin + heparin (▽), plasmin + antithrombin III + heparin (▲)

other hand, plasmin inactivation was enhanced by 11-fold, but in the presence of thrombin it was only 5.6-fold.

Since plasmin inactivation by antithrombin III in the plasma takes place in the presence of fibrinogen, we examined its possible effect on the enzyme-inhibitor reaction. The results (Fig. 2a) indicate that fibrinogen, even at a concentration of 6-fold less than that of the physiological one, diminished the interaction between plasmin and antithrombin III. However, in the presence of high concentration of heparin (0.5 μM), plasmin inactivation by antithrombin III was enhanced suggesting that heparin released plasmin from the plasmin-fibrinogen complex. To understand the relation between fibrinogen and heparin, we examined also the concentration dependence of their effects on plasmin inactivation by antithrombin III. As it can be seen from Figs 3 and 4, fibrinogen, at a concentration of 2.5 mg/ml, inhibited totally plasmin inactivation, whereas heparin, at a concentration of 25 μg/ml, restored plasmin sensitivity to antithrombin III.

In another set of experiments, the effect of fibrin on the plasmin-antithrombin III reaction was investigated. ^{125}I-labelled fibrin was incubated with plasmin and antithrombin III and heparin. At various intervals, aliquots of the reaction mixtures were taken for the determination of radioactivity released. As it can be seen

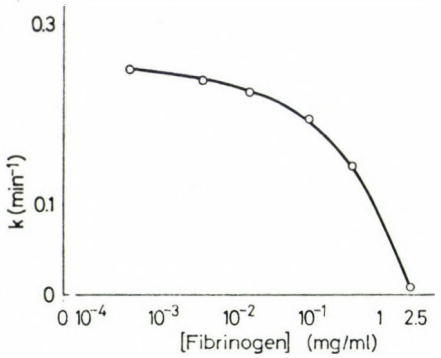

Fig. 3. Dependence of plasmin inactivation by antithrombin III on the concentration of fibrinogen. 0.58 μM plasmin was incubated with 5 μM antithrombin III and various concentrations of fibrinogen in the presence of 30 mM sodium phosphate buffer, pH 7.4 containing 0.11 M NaCl. At different periods, remaining plasmin activity was determined, and first-order rate constant was calculated

from Fig. 2b, antithrombin III was able to inhibit fibrinolysis only in the presence of relatively high concentration of heparin. These data are in accordance with the findings above.

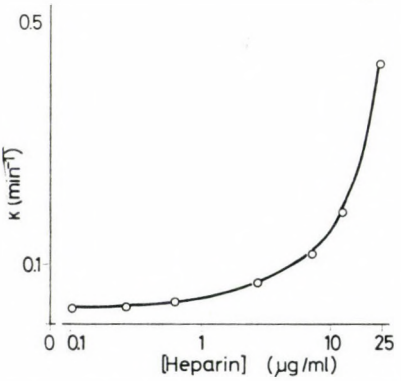

Fig. 4. Dependence of plasmin inactivation by antithrombin III on the concentration of heparin in the presence of fibrinogen. 0.58 μM plasmin, 1.7 μM antithrombin III and 1.1 μM fibrinogen were incubated in the presence of various concentrations of heparin in 30 mM sodium phosphate buffer, pH 7.4 containing 0.11 M NaCl. At different periods, remaining plasmin activity was determined and first-order-rate constant was calculated

Discussion

It is generally believed that antithrombin III acts primarily through the enzymes of blood coagulation, and its influence on the fibrinolytic system, i.e. the inactivation of plasmin, is negligible. In our present work, we demonstrate that

it is very probably the case, especially when plasmin is bound to fibrin or fibrinogen. Namely, whereas thrombin is inactivated easily by antithrombin III, plasmin activity is not influenced by this inhibitor in the presence of fibrinogen or fibrin.

However, when heparin is also present, the sensitivity of these two enzymes are changed. At low concentrations of heparin, at a catalytic amount, only the inactivation rate of thrombin is increased when thrombin and plasmin are together. At high concentration of heparin, at a therapeutical level, heparin makes also bound-plasmin available for antithrombin III. From these data, we conclude that one should consider the risk of the inhibition of fibrinolysis at high dose heparin therapy.

Acknowledgement

We thank Mrs Vera Pichler and Ms Katalin Molnár for technical assistance. This work was supported in part by the Hungarian Ministry of Health, Grant 17/1—10/349 and TPB 70.270/82. Antithrombin III used in these studies was provided by the American Red Cross National Fractionation Center under National Institutes of Health Grant HL 13881.

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Pyrimidine Salvage Enzymes in Human Tonsil Lymphocytes. I. Separation and Properties of Thymidine Kinase Isoenzymes

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Two forms of deoxythymidine kinase were separated from human tonsillar lymphocytes on DEAE-Sephadex column (peak 1 and peak 2 isoenzymes) and identified by gel electrophoresis. Both isoenzymes were found in freshly prepared lymphocytes as well as in lymphocytes cultured in the presence or absence of phytohaemagglutinin, but freshly prepared and phytohaemagglutinin-stimulated cells contained higher activity of peak 1 isoenzyme than the cultured, non-stimulated ones.

Kinetic properties and feed-back inhibition of the two separated isoenzymes were compared. The apparent K_m values of isoenzyme 1 and 2 were 4 μM and 5 μM for thymidine and were 0.15 mM and 0.12 mM for ATP, respectively. Isoenzyme 1 was found to be more sensitive to heat denaturation at 55 °C, and to feedback inhibition by dTTP than isoenzyme 2. On the contrary, isoenzyme 1 was more resistant to dCTP inhibition than the other one.

It is concluded that the isoenzyme 1 activity correlates with the synthesis of DNA, while the activity of isoenzyme 2 seems to be constant. The high activity of isoenzyme 1 in freshly isolated tonsil lymphocytes supports their active proliferation in the organ.

Mitogen stimulation with phytohaemagglutinin is the most frequently used way to induce lymphocytes to proliferate in vitro (Nowell, 1960). This stimulation process involves an increase of DNA synthesis, paralleled by activation of several enzymes involved in either DNA replication (Loeb et al., 1968; Tyrsted et al., 1973) or synthesis of precursors for DNA replication (Loeb et al., 1970; Pegorago and Bernengo 1971; Barlow and Ord, 1975; Piper et al., 1980).

Tonsil lymphocytes have been characterized as cells stimulated in vivo (Staub et al., 1976, 1978, 1982; Staub and Antoni, 1978; Sasvári-Székely et al., 1983), by a high rate of ^{3}H-uridine (Staub et al., 1976; Staub and Antoni, 1978) and ^{3}H-thymidine incorporation (Staub et al., 1978), a high level of DNA polymerase and thymidine kinase activity (Staub et al., 1982). These data together with the flow cytometric analysis indicate that the tonsillar lymphocytes seem to be in the early S phase of the cell cycle (Sasvári-Székely et al., 1983).

It has been hypothesized that the activity of thymidine kinase is closely related to the cell proliferation, since elevated enzyme level has been found in regenerating, and fetal rat tissues (Machovich and Greengard, 1972; Klemperer and Haynes 1968), various neoplastic rat (Hashimoto et al., 1969) and human (Gordon et al., 1968) tissues and stimulated lymphocytes (Loeb et al., 1970; Pegorago and Bernengo 1971; Barlow and Ord, 1975; Piper et al., 1980). Other evidence for this assumption was provided by studies on mitogen stimulated lymphocytes (Munch-Petersen and Tyrsted, 1977) and other dividing cells (Okuda et

al., 1972; Hatanaka and Dulbecco, 1967; Görbner, 1979; Yamada et al., 1979, 1980; Lee and Cheng, 1976; Adler and McAuslan 1974; Caron and Unsworth, 1978; Nawata and Kamiya, 1975; Kit, 1976; Taylor et al., 1972). It was found that mitogen stimulation of blood lymphocytes was paralleled by the appearance of a new highly active isoenzyme (Munch-Petersen and Tyrsted, 1977). These facts suggest a close correlation between DNA synthesis and the appearance of this isoenzyme.

The present communication deals with the separation of thymidine kinase isoenzymes from tonsillar lymphocytes and with comparison of kinetic and regulatory proporties of partially purified isoenzymes.

Materials and methods

Cell source

Tonsillar lymphocytes were obtained from surgically removed tonsils of 3–6 year-old children as described previously (Staub et al., 1976). Tonsillar lymphocytes were used immediately for enzyme preparation (extract of freshly prepared cells) or were cultured for 48 h in the presence or absence of phytohaemagglutinin as described earlier (Staub et al., 1982).

Materials

DEAE-Sephadex A-25 was purchased from Pharmacia (Uppsala, Sweden), Phytohaemagglutinin (P grade) from Difco Laboratories (Detroit, Mich., U.S.), and DEAE-cellulose paper (Whatman DE-81) was obtained from W. and R. Balston Ltd. (Springfield Mill, Maidstone, Kent, England). Unlabelled deoxyribonucleosides and deoxyribonucleotides were purchased from Sigma Chemical Co. (St. Louis, M., U.S.). ^{3}H-thymidine (spec. act. 96.2×10^{10} Bq, i.e. 26 Ci/mmol) from the Institute of Isotopes (Prague, Czechoslovakia), the other reagents were from Reanal (Budapest, Hungary).

Preparation of cell extract

The cell suspensions were centrifuged at 500 *g* for 10 min and the cell pellet was drained and resuspended at a cell concentration of 0.5×10^8–2×10^8 cells per ml in a 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MgCl_2$ and 1 mM dithiothreitol (called buffer H). The cells were disrupted with a motor-driven, tightly fitting teflon pestle in a glass homogenizer three times, for 2 min. The homogenate was centrifuged for an hour at 105 000 *g*. The supernatant was used as crude extract of the cells.

DEAE-Sephadex chromatography

The crude extract was fractionated by adding saturated $(NH_4)_2SO_4$ (pH 7.5) to 50% saturation, and the mixture was stirred for 20 minutes. After centrifugation at 10 000 *g* for 20 min, the precipitate was dissolved in 5 volumes of buffer H, brought to 25% saturation of $(NH_4)_2SO_4$, and left for two hours at 0 °C. The pre-

cipitate was then centrifuged at 10 000 *g* for 20 min, and the supernatant was extensively dialyzed against buffer H. The dialysate was centrifuged again at 10 000 *g* for 20 min, and the supernatant was loaded onto a DEAE-Sephadex A-25 column (1 × 30 cm) previously equilibrated with the same buffer. Gradient elution from 0.0 to 0.4 M KCl, or stepwise elution (0.015 M and 0.4 M) was used as indicated in the legends. Fractions of 3 ml were collected. All procedures were performed at 0 to +4 °C.

Non-denaturing polyacrylamide gel electrophoresis and in situ determination of enzyme activity

Slab-gel electrophoresis was performed under non-denaturating conditions (Mechali et al., 1979) at 0–+2 °C. The lower gel was polymerized from acrylamide (5%), bisacryl-amide (0.6%), N,N,N',N'-tetramethyl ethylenediamide (0.05%) and $K_2S_2O_3$ (0.05%) containing 50 μM thymidine, 2 mM $MgCl_2$, 0.125 M Tris-HCl (pH: 8.5) and 20% glycerol. The upper gel was basically the same as the lower gel except that the buffer was 0.025 M Tris-HCl (pH: 8.5). Samples (50 to 100 μl) were layered onto the holes of the slab together with bromophenol blue as marker. The electrophoresis was performed at constant voltage of 150 V for approximately 6 h (5–15 mA). The running buffer contained 0.05 M Tris-HCl, 0.12 M glycine (pH: 8.5), 2 mM $MgCl_2$, and 17% glycerol. At the end of the run, the slab was removed and placed onto DEAE-cellulose paper having the same size (10 × 10 cm). A thymidine kinase reaction mixture (2.5 ml) was pipetted onto the DEAE paper, and the whole was hermetically sealed for 3 h of incubation at 37 °C. After incubation, the sheet of DEAE paper was washed as described under "Enzyme assay", dried, sliced into 1 cm^2 pieces and measured for radioactivity in a toluene based cocktail.

Enzyme assay

Thymidine kinase activity was assayed by the method of Bresnik et al. (1967) with some modifications as described previously (Staub et al., 1982). The following composition of the standard reaction mixture was found to be optimal for the determination of enzyme activity: 100 mM Tris-HCl buffer, pH 7.5 (20 °C), 10 mM $MgCl_2$, 10 mM NaF, 10 mM ATP (neutralized), 25 μM ^{3}H-thymidine (spec. act. 1.6×10^{15} Bq/mol) and 80–100 μg protein of crude extract in a final volume of 200 μl. After preincubation of the reaction mixtures for 5 min at 37 °C the reaction was started by addition of enzyme, and terminated by the application of the reaction solution on paper squares. Unphosphorylated thymidine was eluted by washing the paper squares three times for 10 min in 5 mM ammonium formate, pH 4 (20 ml per square) and in water and ethanol for 5 min, respectively. The paper squares were dried and the radioactivity was determined by liquid scintillation spectrometry. In all experiments the enzyme activities were calculated from initial velocities, i.e. the reaction rate was proportional to the amount of enzyme and to the time of incubation.

Definition of the unit of enzyme activity

The enzyme activity is expressed as nmoles of deoxynucleoside phosphorylated per hour at 37 °C. The specific activity is defined as nmoles of deoxynucleoside phosphorylated per hour per milligram of protein as determined by the method of Braedford et al. (1976) using bovine serum albumin as standard.

Results

Separation of thymidine kinase isoenzymes by DEAE-Sephadex-chromatography and by polyacrylamide gel electrophoresis

Figure 1 shows the comparison of chromatographic profile of extracts from freshly prepared tonsillar lymphocytes (A) and cells cultured for 48 h at 37 °C in the absence (B) or presence (C) of phytohaemagglutinin. As can be seen, for all types of tonsillar lymphocytes that were measured, the thymidine kinase activity

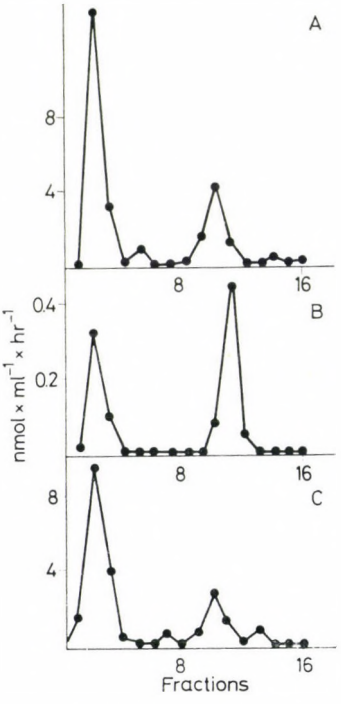

Fig. 1. Separation of thymidine kinase isoenzymes by DEAE-Sephadex chromatography. Freshly prepared tonsillar lymphocytes (A) and cells cultured for 48 h at 37 °C in the presence (C) or absence (B) of phytohaemagglutinin were applied to a DEAE-Sephadex A-25 column, and a KCl gradient elution was used as described under "Methods". Enzyme activity of thymidine kinases was measured in the eluted fractions, and expressed as nmoles of phosphorylated thymidine per ml of fraction per hour of incubation at 37 °C

Table 1

Purification of thymidine kinase

Procedure	Protein mg/ml	Activity nmol/ml/h	Total activity nmol/h	Specific nmol/mg/h
I. Homogenate	7.8	2.5	39.0	0.32
II. 100 000 *g* supernatant	3.4	5.0	36.6	1.47
DEAE-Sephadex peak 1	1.9	8.6	20.6	4.52
DEAE-Sephadex peak 2	0.9	6.15	10.3	6.84

was separated into two parts. The enzyme activity found under peak 1 was 2.5-fold higher than the activity found under peak 2 in the shown experiment (Fig. 1A) and it ranged from 2 to 4-fold depending on the tonsils used for cell preparation. For unstimulated lymphocytes (Fig. 1B) the activity of both isoenzymes was very low as compared with the freshly prepared tonsillar cells (Fig. 1A) and to stimulated ones (Fig. 1C). The purification procedure is summarized in Table 1. As a result of a typical separation procedure, a purification of 14-fold and 30-fold were achieved for peak 1 and peak 2 isoenzymes, respectively, with an overall recovery of 79%.

To identify the separated isoenzymes, polyacrylamide gel electrophoresis was performed and the enzyme activity was measured on the gel slabs (Fig. 2). Peak 1 gave a single spot ($R_F = 0.17$), and peak 2 rise also to a single spot ($R_F = 0.44$) of the enzyme activity. Both of these spots could be found in crude extracts of freshly prepared tonsillar lymphocytes.

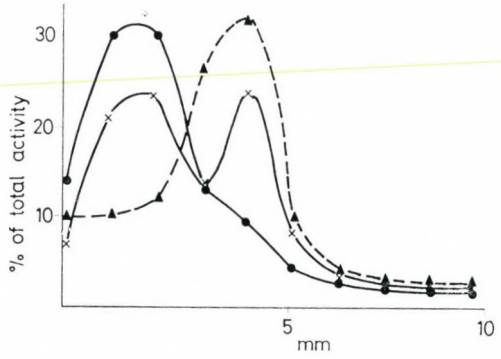

Fig. 2. Non-denaturating polyacrylamide gelelectrophoresis of thymidine kinase isoenzymes Tonsillar thymidine kinase isoenzymes were separated on DEAE-Sexphadex column (see Fig. 1), and the separated isoenzymes were applied to polyacrylamide gel slabs, and electrophoresis was performed as described under "Methods". (●–●): DEAE-Sephadex peak 1; (▲–▲): DEAE-Sephadex peak 2; (×–×): unseparated extract of lymphocytes. Results are expressed as the percentage of the total activity of one type of applied enzyme for better comparison

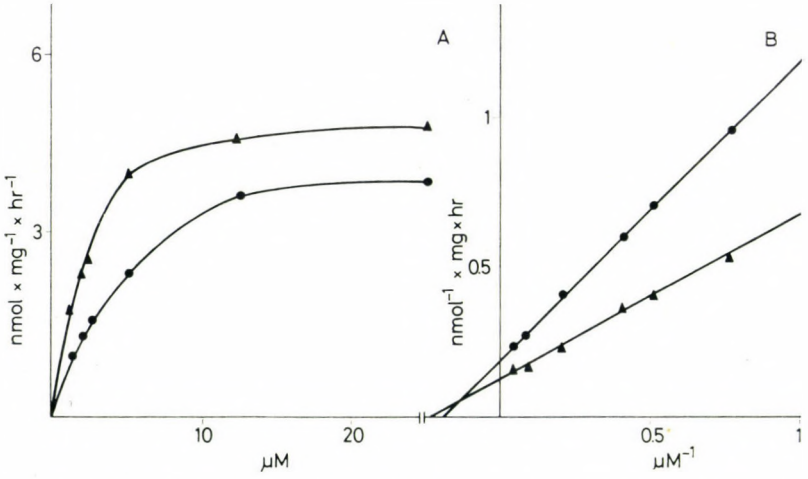

Fig. 3. Effect of thymidine concentration on the initial reaction rate of separated thymidine kinase isoenzymes. Initial enzyme activity was measured in a standard reaction mixture except that concentration of thymidine was varied as indicated. A: thymidine saturation plot of the data; B: double-reciprocal plotting; (●–●) peak 1; (▲–▲) peak 2 isoenzymes

Comparison of kinetic and regulatory properties of thymidine kinase isoenzymes derived from freshly isolated tonsillar lymphocytes

Initial velocity studies were performed by keeping one substrate at a fixed concentration and varying the concentration of the other substrate. For both isoenzymes, linear plots were obtained when 1/v was plotted against 1/substrate concentration (see Fig. 3). The apparent K_m for thymidine was 4 μM and 5 μM for peak 1 and peak 2 isoenzymes, respectively, and the same values for ATP were 0.15 mM and 0.12 mM for isoenzymes 1 and 2, respectively (see Fig. 4). All K_m values were determined at the saturation level of the other substrates.

The investigation of regulatory properties of the separated isoenzymes included inhibition studies by either dTTP or dCTP. The results are summarized

Table 2

Feed-back inhibition of thymidine kinase isoenzymes

Inhibitor	Conc. mM	Enzyme activity, %	
		peak 1	peak 2
dTTP	0.01	79	97
	0.10	32	50
	1.00	8	14
dCTP	0.01	109	96
	0.10	116	64
	1.00	87	57

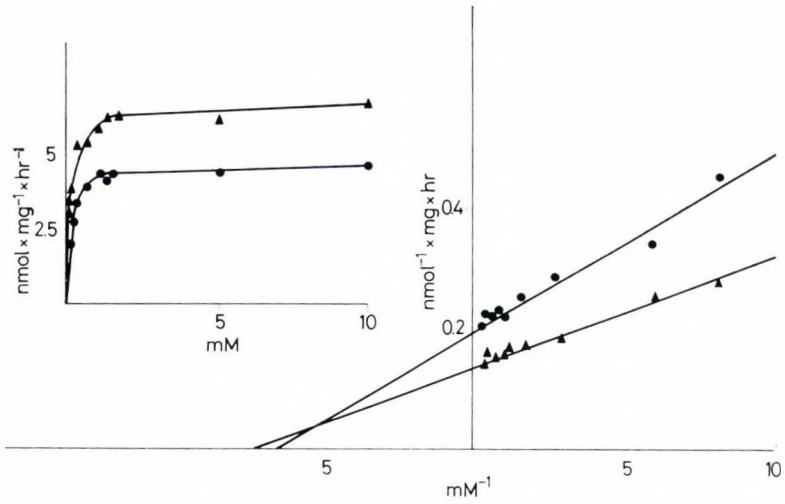

Fig. 4. Effect of ATP concentration on the initial rate of separated thymidine kinase isoenzymes. Initial enzyme activities were measured in a standard reaction mixture except that concentration of ATP was varied as indicated: double reciprocal plotting; insert: Michaelis-Menten plot of the data; (●—●) peak 1; (▲—▲) peak 2 isoenzymes

in Table 2. At saturating level of ATP, and with thymidine as a variable substrate, dTTP was found to inhibit both isoenzymes in a noncompetitive manner, while a mixed inhibition was obtained with ATP as the variable substrate (data not shown).

Heat-inactivation experiments revealed a significant difference between the heat-stability of the two isoenzymes, i.e. isoenzyme 1 was found to be far more sensitive to heat-inactivation at 55 °C than isoenzyme 2 (see Fig. 5).

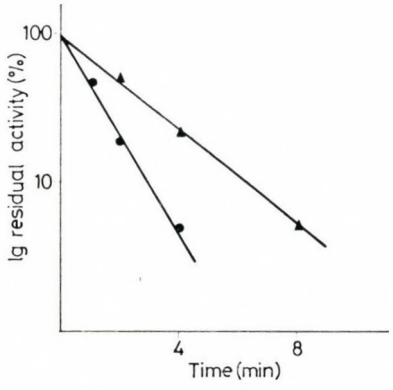

Fig. 5. Heat inactivation of separated thymidine kinase isoenzymes. Initial enzyme activity was measured in a standard reaction mixture after incubation at 55 °C for the indicated times (●—●) peak 1; (▲—▲) peak 2 isoenzymes

Discussion

Thymidine kinase activity has been shown to increase in human lymphocytes during stimulation by phytohaemagglutinin (Loeb et al., 1970; Pegorago and Bernengo, 1971; Barlow and Ord, 1975; Staub et al., 1982). The high level of this enzyme has been reported in freshly prepared tonsillar lymphocytes without mitogen stimulation (Staub et al., 1982). Moreover, DNA polymerase α an other enzyme involved in DNA replication process, has also been shown to exhibit a high activity in these cells (Staub et al., 1982). Consequently, freshly prepared tonsillar lymphocytes have a high rate of DNA synthesis as it was suggested first by the high rate of ^{3}H-thymidine incorporation (Staub et al., 1976; Staub and Antoni, 1978; Sasvári-Székely et al., 1983). This "spontaneous" DNA synthesis decreased with time during incubation in vitro without mitogen stimulation (Staub and Antoni, 1978).

The pronounced increase of thymidine kinase activity during stimulation of peripheral blood lymphocytes has been considered to reflect the induction of a new isoenzyme, specific for dividing cells (Munch-Petersen and Tyrsted, 1977). The presence of thymidine kinase isoenzymes has also been reported in tissues with rapid cell proliferation (Okuda et al., 1972; Hatanaka and Dulbecco, 1977; Görbner, 1979; Yamada et al., 1979, 1980; Lee and Cheng, 1976; Adler and McAuslan, 1974; Caron and Unsworth, 1978; Nawata and Kamiya, 1975; Kit, 1976; Taylor et al., 1972). Munch-Peterson and Tyrsted have separated two thymidine kinase isoenzymes from mitogen stimulated blood lymphocytes, using DEAE-Sephadex chromatography (Munch-Petersen and Tyrsted, 1977). The appearance of isoenzyme 1 coincided with DNA synthesis of the cells (Munch-Petersen and Tyrsted, 1977). In the present study separation of two thymidine kinase isoenzymes from freshly isolated and mitogen stimulated tonsillar lymphocytes was achieved also by DEAE-Sephadex chromatography. The existence of two isoenzymes was also demonstrated by polyacrylamide gel-electrophoresis. About 70% of the total thymidine kinase activity of freshly isolated and in vitro stimulated tonsillar lymphocytes appeared in peak 1. This finding also suggests, in accordance with our previous results (Staub et al., 1978; Staub and Antoni, 1978; Staub et al., 1982; Sasvári-Székely et al., 1983), that human tonsillar lymphocytes may regarded as lymphocytes proliferating in the organ.

Isoenzyme 1 from freshly isolated tonsillar lymphocytes has similar properties to those of the same isoenzyme in mitogen stimulated lymphocytes (Munch-Petersen and Tyrsted, 1977) and also to isoenzymes from various replicating cells (Hatanaka and Dulbecco, 1967; Görbner, 1979; Yamada et al., 1979, 1980; Lee and Cheng, 1976; Adler and McAuslan, 1974; Caron and Unsworth, 1978; Natawa and Kamiya, 1975; Kit, 1976; Taylor et al., 1972). The similar properties are; the slow migrating species under electrophoresis (Lee and Cheng, 1976; Adler and McAuslan, 1974; Caron and Unsworth, 1978), a relatively high sensitivity to heat denaturation (Hatanaka and Delbecco, 1967; Taylor et al., 1972), and a low K_m value for thymidine. The regulatory properties of these isoenzymes are also

similar in respect to the feed-back inhibition by dTTP (Munch-Petersen and Tyrsted 1977; Yamada et al., 1979; Lee and Cheng, 1976; Ellimns, 1982), and in resistance to dCTP (Yamada et al., 1979, 1980; Lee and Cheng, 1976; Nawata and Kamiya, 1975; Taylor et al., 1972; Ellimns et al., 1982).

Comparing isoenzyme 2 from tonsillar lymphocytes to the same isoenzyme from mitogen induced blood lymphocytes (Munch-Petersen and Tyrsted, 1977), differences were found in respect to their feed-back inhibition. The tonsillar isoenzyme 2 is more sensitive to dTTP inhibition and it could also be inhibited by dCTP similarly to isoenzyme 2 from other sources (Yamada et al., 1979, 1980; Lee and Cheng, 1976; Nawata and Kamiya, 1975; Taylor et al., 1972) but not to isoenzyme 2 from in vitro stimulated blood lymphocytes (Munch-Petersen and Tyrsted, 1977).

Such difference in the feed-back inhibition of thymidine kinase isoenzymes derived from in vivo stimulated human tonsillar or from in vitro stimulated peripheral lymphocytes is difficult to interpret. Moreover, difference was found only in the case of isoenzyme 2, while the properties of isoenzyme 1 were very similar in both types of lymphocytes.

Recently, evidences are accumulating about differences in nucleoside salvage metabolism of various types of lymphocytes. Autoradiographic studies revealed different labelling of mature and immature lymphocytes with ^{3}H-deoxycytidine and ^{3}H-thymidine (Osogoe and Ueki, 1970; Hamatani and Amano, 1980; Amano and Everett, 1976), and the level of these nucleoside kinases were found to be different in various lymphoid tissues (Staub et al., 1983).

Findings concerning the regulation of the activity of thymidine kinase isoenzymes and further investigations about other nucleoside kinases in lymphocytes might contribute to a better understanding of the nucleotide metabolism and its regulation in different lymphoid tissues and its importance in the lymphocyte differentiation.

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Pyrimidine Salvage Enzymes in Human Tonsil Lymphocytes: II. Purification and Properties of Deoxycitidine Kinase

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Human tonsillar lymphocytes incorporate about 7–10 times more [^{3}H]-deoxythymidine than [^{3}H]-deoxycytidine at the same extracellular nucleoside concentration, and both incorporations could be inhibited by 10^{-5} *M* arabinosyl-cytosine to 99%. On the other hand, the crude extract of these cells had about 10 times more deoxycytidine kinase than deoxythymidine kinase specific activity, as it was reported previously as well. Therefore, the differences in the labelling of these cells by [^{3}H]-deoxycytidine and [^{3}H]-thymidine cannot be simple due to the different levels of phosphorylating enzymes in the cells.

The deoxycytidine kinase of human tonsillar lymphocytes was purified 38.6-fold by DEAE-Sephadex chromatography, and some kinetic and regulatory properties of the purified enzyme were investigated. Deoxycytidine kinase was eluted as a single peak from DEAE-Sephadex column and also from Sephadex G-100 column indicating a molecular weight of about 60 000; no isoenzymes could be detected. During the purification procedure an increase of the enzyme activity was observed suggesting the inhibition of the enzyme in the cells. An apparent K_m of 13 μM for deoxycytidine and K_i of 55 μM for arabinosyl-cytosine were found for the tonsillar deoxycytidine kinase. The enzyme was strongly inhibited by dCTP, but not by the other deoxyribonucleoside triphosphates.

One of the commonly used method for measuring DNA synthesis of lymphocytes is the labelling of cells with radioactive thymidine. However, there are some types of lymphocytes, which could be labelled more intensively with radioactive deoxycytidine than with thymidine (Hamatani and Amano 1980). Both pyrimidine nucleosides enter into the nucleotide pools via the salvage pathways, i.e. transport into the cells and subsequent phosphorylation, and the later process was shown to be the rate limiting step of incorporation (Plagemann et al., 1978). The enzymes, phosphorylating these nucleosides, are thymidine kinase (EC 2.7.1.21.) and decxycytidine kinase (EC 2.7.1.74.), the latter found primarily in lymphoid tissues (Durham and Ives, 1969). Previous results in other (Durhan and Ives, 1969) and in our laboratory (Staub et al., 1983) have shown that the amounts of these two kinases vary widely in different lymphoid organs but the cause of these variations is unknown.

The questions are also of practical interest, because deoxycytidine kinase has an important role in the activation of the chemotherapeutic agent, arabinosyl-

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cytosine araC (Mejer, 1982; Cohen, 1966), being widely used in the treatment of acute leukemia. This analogue of deoxycytidine acts primarily as an inhibitor of DNA polymerase α (for review see Cozzarelli, 1977) after phosporylation by deoxycytidine kinase. On the other hand, araC has also prevented the mitogen induced increase of thymidine kinase and DNA polymerase α as it was shown previously in tonsillar lymphocytes (Staub et al., 1982).

A series of investigations of deoxycytidine kinase isolated from thymus, as a T lymphocyte source, were reported (Ives and Durham, 1970; Durham and Ives, 1970; Baxter et al., 1978; Momparler and Fischer, 1968). Deoxycytidine kinase was also isolated from the leukemic cell line L 1210 (Chang et al., 1982; Kessel, 1968) used widely for evaluation of antitumor agents (Kessel, 1968) properties of deoxycytidine kinase originated from T and B lymphoblastoid cell lines raised the possibility that there exist T and B cell specific isoenzymes (Hershfield et al., 1982). In order to clarify whether the properties of deoxycytidine kinase are different in various organs, it is desirable to investigate purified enzymes of different origin. In this paper we describe the partial purification and some properties of deoxycytidine kinase from human tonsils, which can be regarded as a natural source of B lymphocytes (Staub et al., 1978).

The purification and properties of human tonsillar thymidine kinase isoenzymes are described in a separate paper (Sasvári-Székely et al., 1983).

Materials and methods

DEAE-Sephadex A-25 and Sephadex G-100 was purchased from Pharmacia, Uppsala, Sweden; DEAE-cellulose paper (Whatman DE-81) from W. and R. Balston Ltd., Springfield Mill, Maidstone, Kent, England. Unlabelled deoxyribonukleosides and deoxyribonucleotides as well as dithiothreitol were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S., [^{3}H]-thymidine from the Institute of Isotopes Prague, Czechoslovakia, [^{3}H]-deoxycytidine from Radiochemical Centre, Amersham, Bucks., U.K.; (spec. act. 96.2×10^{10} Bq each, i.e. 26 Ci per mmole); Dextran Blue-, bovine serum albumin-, RNase A were products of Pharmacia; for enzyme concentration Minicon B15 (Amicon) was used. The other reagents were from Reanal, Budapest, Hungary, and all were analytically pure.

Cell source

Tonsillar lymphocytes were obtained from surgically removed tonsils of 3–6 year-old children as described previously (Staub et al., 1976), and aliquots of isolated lymphocytes were used immediately for the measurement of incorporation. The rest of the cells were stored at −20 °C until used for enzyme preparation. The storage of the cells had no effect on the yield of enzyme activity.

Measurement of [^3H]-thymidine and [^3H]-deoxycytidine incorporation

The incorporation of labelled nucleosides into the intact cells was measured immediately after preparing tonsillar lymphocytes, in short term cultures (2×10^6 cells per 1 ml of Eagle medium). The incorporation of labelled nucleosides into the DNA fraction of the cells was performed as described previously (Staub et al., 1982). Briefly: after labelling the cells with [^{3}H]-thymidine or [^{3}H]-deoxycytidine (55 kBq, i.e. 1.5 μCi per ml; specific radioactivity: 10^4 cpm per pmol) for an hour at 37 °C, the cells were washed with Eagle medium containing an excess of non-labelled nucleosides. Then the cell pellet was homogenized in 0.5 N perchloric acid and left for 20 min at 0 °C. After washing twice with cold 0.5 N perchloric acid, the insoluble fraction of cells was hydrolyzed in the same acid for 30 min at 90 °C, and aliquots were assayed for radioactivity in a toluene based coctail.

Determination of the enzyme activity

Enzyme activity was measured by the ion-exchange paper method of Ives et al. (1969) essentially in the same manner as described previously (Staub et al., 1983) except that an excess of nonlabelled deoxycytidine was added (final concentration: 500 μM) at the end of the incubation period. Stored enzymes were reactivated by incubation with a buffered dithiothreitol and bovine serum albumin, for a hour at room temperature before kinetic studies were carried out (Ives and Durham, 1970). The reaction mixture contained: ATP 10 mM; $MgCl_2$ 12 mM; Tris-HCl buffer pH 7.5, 50 mM; [^{3}H]-deoxycytidine 29 mM (86.5 cpm per pmole) and enzyme containing bovine serum albumin and dithiothreitol at concentrations of 250 μg/ml and 12.5 mM, respectively. The reaction rate showed a linear relation to the incubation time and to the amount of the protein. The incubation time was 10 min for crude extract and 40 min for purified enzymes.

Definition of the unit of enzyme activity

The enzyme activity is expressed as nmoles of deoxynucleoside phosphorylated per minute at 37 °C. The specific activity is defined as nmoles of deoxynucleoside phosphorylated per minute per milligram of protein as determined by the method of Bradford (1976), using bovine serum albumin as standard.

Enzyme purification

The temperature was maintained at 0–4 °C throughout the whole procedure. Tonsillar lymphocytes (4×10^9/cells) were suspended in 10 ml of 50 mM Tris-HCl, pH 7.5 containing 10 mM KCl; 5 mM $MgCl_2$; 1 mM dithiotreitol, left for 10 min and were homogenized with a motor driven teflon pestle in a glass homogenizer. The homogenate was centrifuged at 100 000 *g* for an hour. The supernatant called "crude extract", was dialysed against the column buffer (see below) to remove KCl. In some experiments lymphocyte homogenization was performed in KCl-free buffer and in this case dialysis was omitted.

The crude extract was applied onto a DEAE-cellulose A-25 column (0.75×4 cm) previously equilibrated with 50 mM imidazol buffer (pH 7.0) containing 5 mM

Table 1

Purification of human tonsillar deoxycytidine kinase

		Protein mg/10^6 cells	Specific activity nmoles/mg/min	Yield %	Purification (-fold)
I.	Crude extract with 0.1 M KCl	54	0.22	100	1
	Step-wise elution from DEAE-Sephadex column		1.07	268	4.8
II.	Crude extract without KCl	15.3	0.78	99	3.5
	Gradient elution from DEAE-Sephadex column		8.5	202	38.6

$MgCl_2$; 4 mM β-mercaptoethanol and 20% ethyleneglycol. Elution was started with the same buffer followed either by a linear 0–1.0 M KCl gradient (see Fig. 2) or by a step-wise elution with the same buffer containing 0.4 M KCl (see Table 1). Other details are given in the legends to the Figures. Fractions exhibiting enzyme activity were called as "partially purified enzyme" and used for inhibition experiments.

Sephadex gel-filtration

Sephadex G-100 column (60 × 1 cm) was equilibrated with 0.1 M, pH 7.4 TRIS-HCl buffer, containing 0.05 M KCl, 1 mM $MgCl_2$ and 5 mM β-mercaptoethanol. Dextran Blue was used to determine the exclusion volume; bovine serum albumin, and RNase A were used as markers of molecular weight. Marker proteins were determined by their optical density at 280 nm, and enzyme activity was measured in each fraction.

Results

Comparison of phosphorylation of [^3H]-deoxycytidine by intact tonsillar lymphocytes and by the crude extracts of the same cells

Lymphocytes isolated from human tonsils were labelled either with [^{3}H]-thymidine or with [^{3}H]-deoxycytidine, having the same specific activity, for an hour at 37 °C (Fig. 1A). The labelling of the cells was measured either in the presence or in the absence of 10^{-5} arabinosyl-cytosine. Cell-free extract of the same cells was made (see Methods), and the activity of deoxycytidine kinase and thymidine kinase was determined (Fig. 1B). As it can be seen, tonsillar lymphocytes incorporated far more [^{3}H]-thymidine than [^{3}H]-deoxycytidine into their DNA. Both incorporations could be inhibited by araC. However, determination of the level of the kinases from the crude extract of the same cell population showed a reversed ratio of these enzymes: the activity of deoxycytidine kinase was ten times more

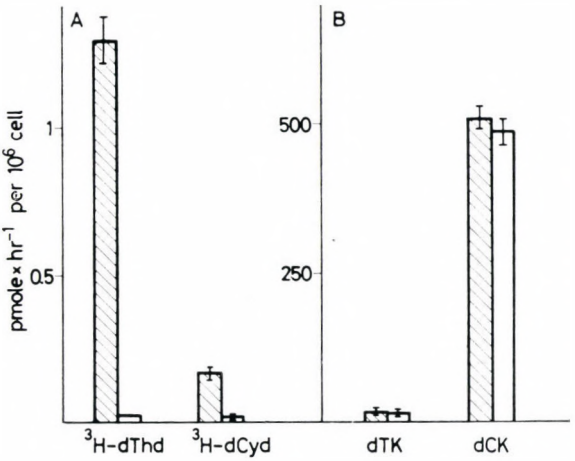

Fig. 1. Phosophorylation of [^{3}H]-thymidin and [^{3}H]-deoxycytidine by intact tonsillar lymphocytes (A) and by the crude extract of the same cells (B). Aliquots of freshly prepared tonsillar lymphocytes (2×10^6 cells per ml) were labelled with either [^{3}H]-thymidine or [^{3}H]-deoxycytidine, as indicated, in the presence (blank bars) or absence (dashed bars) of 10^{-5} M araC for an hour at 37 °C. The incorporated radioactivity into the DNA was determined as described under "Methods", the results are expressed as incorporated pmoles per hour of 10^6 cells. The rest of the cells were used for preparation of the crude extract (see "Methods") and the deoxycytidine kinase and thymidine kinase activities were determined under optimal conditions (incubation time 10 min, amount of enzyme was 5 μl of the extract), in the presence (blank bars) or absence (dashed bars) of 10^{-5} M araC. The enzyme activity is expressed as pmoles of phosphorylated nucleoside per hour per crude extract of 10^6 cells. In all cases the mean values of three parallel determinations and the standard error are shown

han the activity of thymidine kinase and none of the enzyme activities were affected by 10^{-5} M of arabinosyl-cytosine.

Partial purification of tonsillar deoxycytidine kinase on DEAE-Sephadex column

For better comparison of the kinases, an attempt was made to purify deoxycytidine kinase in the same way as thymidine kinase was purified (Sasvári-Székely et al., 1985). Applying the crude extract directly onto a DEAE-Sephadex column, the whole activity was retained (see Fig. 2). The enzyme was eluted by a linear gradient of 0–1.0 M KCl or by a stepwise elution with 0.4 M KCl (see Fig. 2 and Table 1). Two types of purification procedures are compared in Table 1. The data obtained show that KCl is not necessary for the extraction of the enzyme. If we used KCl-free extraction buffer, the protein content decreased 3–4 times, resulting a higher specific activity of the crude extract. Using a KCl-free extraction and a gradient elution of the enzyme, a 38.6-fold purification was achieved (see Table 1). An interesting phenomenon of this purification is that enzyme activity increased about twice during the procedure. Since the enzyme activity of the crude extract was measured carefully under optimal conditions (i.e. linearity with protein content

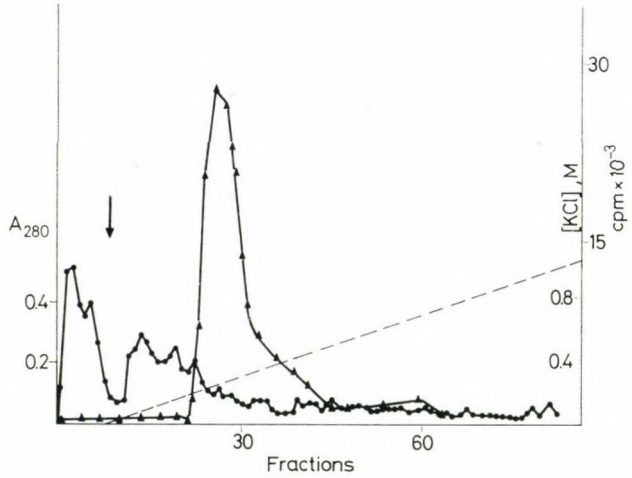

Fig. 2. DEAE-Sephadex A25 chromatography of tonsillar deoxycytidine kinase. Crude extract (10 mg of protein) of cells was loaded onto the column previously equilibrated with 50 mM imidazol buffer pH 7.4 containing 5 mM $MgCl_2$ and 4 mM β-mercaptoethanol, and 20% ethyleneglycol. Elution was carried out first with the equilibration buffer, and next with a linear gradient of KCl (0—1.0 M) as indicated on the figure. Flow rate was 0.2 ml per min and 1 ml fractions were collected. Solid line, deoxycytidine kinase activity (△ . . . △) and UV absorption (· —·); broken line, KCl concentration

and incubation time), the increase of enzyme activity during purification raises the possibility of an inhibitor of the enzyme in the cells.

The stability of the enzyme is far more dependent on sulfhydryl reagents. The purified enzyme lost about half of its activity after the freezing and melting, however, the whole activity of the enzyme can be restored by preincubation of the enzyme with dithiothreitol and bovine serum albumin for an hour at room temperature.

Molecular weight of tonsillar deoxycytidine kinase

The molecular weight of the deoxycytidine kinase was determined on Sephadex G-100 column using appropriate standards (see Fig. 3). A single peak of enzyme activity was found at the same elution volume where the bovine serum albumin was obtained, indicating molecular weight of about 60 000 for human tonsillar deoxycytidine kinase.

Regulatory properties of the tonsillar deoxycytidine kinase

The enzyme was investigated for the inhibition pattern of the known cytostatic agent, araC, and also for the feedback inhibition by deoxyribonucleoside triphosphate (dCTP). Figure 4 shows the percent of inhibition as the function of the concentration of araC and dCTP. Using the partially purified deoxycytidine kinase dCTP was used in each case together with the same amount of $MgCl_2$,

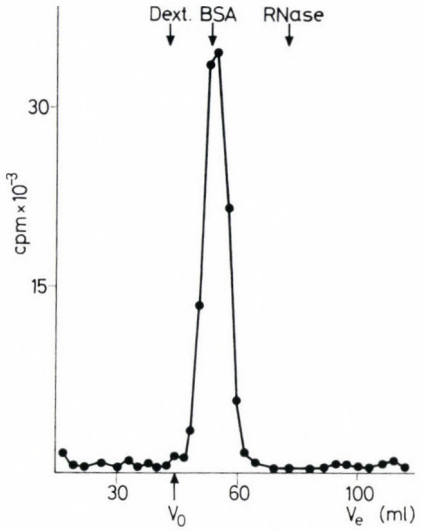

Fig. 3. Sephadex G-100 gel filtration of deoxycytidine kinase. Gel was equilibrated with 0.1 M Tris-HCl buffer pH 7.4, containing 0.05 M KCl, 1 mM $MgCl_2$ and 5 mM mercaptoethanol. Crude extract of the cells previously concentrated by Minicon B 15 (Amicon), was loaded onto the column (25 mg of protein) and eluted with the same buffer at the flow rate of 0.1 ml per min, and 1 ml fractions were collected. Enzyme activity is expressed as the measured cpm for 30 μl aliquots of each fraction (.–.). Elution volume of marker proteins are indicated by arrows. Dext: Dextran Blue, BSA: bovine serum albumine, RNase: ribonuclease A

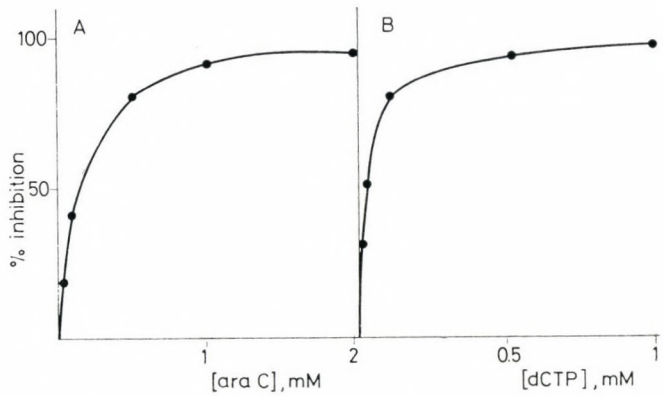

Fig. 4. Inhibition of deoxycytidine kinase by arabinosyl-cytosine (A) and by dCTP (B). The concentration dependence of inhibition by arabinosyl-cytosine (A) as well as by dCTP (B) was measured in a standard reaction mixture containing 2 μg of partially purified enzyme per sample. The results are expressed as the percentage of inhibition. The enzyme activity, measured without inhibitor equalled 2.06 nmoles per mg protein per minute. dCTP was added together with the same amount of $MgCl_2$

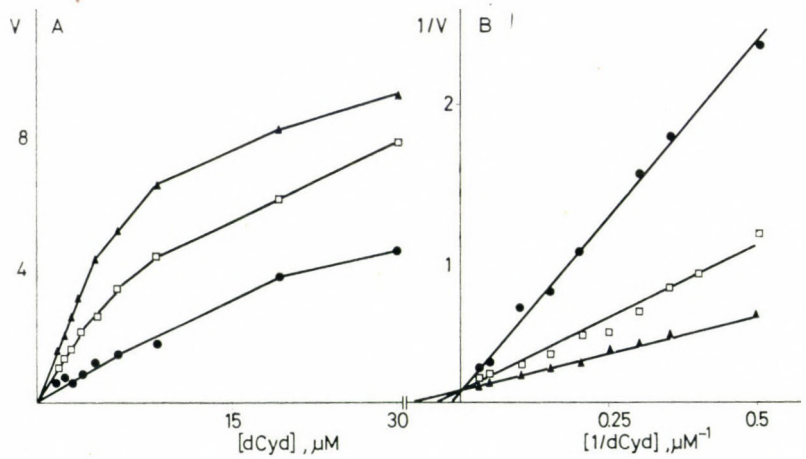

Fig. 5. Effect of varying concentrations of deoxycytidine on the inhibition of arabinosyl-cytosine. Initial reaction rate was measured in the presence of araC with varying concentrations of deoxycytidine with 2 μg of partially purified enzyme incubated for 40 min at 37 °C. (A) Michaelis-Menten plot of the data, (B) double-reciprocal plot. Velocities are expressed as monophosphorylated substrate per mg protein per min. The reaction rate was measured in the presence (□–□: 100 μM; ·–· 250 μM) or absence (▲–▲) of arabinosyl-cystosine

to avoid the apparent inhibition of a nucleoside triphosphate caused by the binding of $MgCl_2$ present in the standard reaction mixture. All the other deoxyribonucleoside triphosphates (dTTP, dGTP, dATP) were also used under the same conditions, but no inhibition was found up to 0.2 mM.

Measuring the initial reaction rate as the function of deoxycytidine concentration, regular Michaelis-Menten kinetics were obtained, with an apparent K_m of 13.3 μM (see Fig. 5). Arabinosyl-cytosine was found to be a competitive inhibitor of the reaction and the apparent K_i for araC was 55 μM (Fig. 5).

Discussion

The incorporation of [^{3}H]-deoxycytidine and [^{3}H]-thymidine has been shown to be different in various areas of lymphoid organs (Hamatani and Amano, 1980). The problem arose whether these differences were due to different kinase activities in these cells. Attempts to solve this problem have been made in the previous (Staub et al., 1983) and in the present study by comparing activities of deoxycytidine and thymidine kinases, the enzymes responsible for the phosphorylation of the labelled nucleosides. However, the differences in the [^{3}H]-deoxycytidine and [^{3}H]-thymidine incorporation into tonsillar lymphocytes cannot be explained by the differences in the amounts of deoxycytidine and thymidine kinase activities demonstrated in extracts of these cells (see Fig. 1). While it is obvious from the data obtained that there is sufficient amount of kinase to phosphorylate the entering [^{3}H]-deoxycytidine, one may speculate about the cause of the difference in the

phosphorylating capacity of intact cells and that of their extracts. One of the most obvious explanations would be the inhibition of the kinase in the cells, presumably by the intracellular dCTP. This conclusion is in accordance with the results of Plagemann et al. (1978). Our data presented here support this explanation since we observed an increase of the enzyme activity during purification (see Table 1), and also dCTP inhibited the purified enzyme (see Fig. 3). However, more detailed studies of nucleoside metabolism of intact cells seems to be necessary to confirm this potential explanation.

The intracellular activity of deoxycytidine kinase has a practical interest as well, since arabinosyl-cytosine is phosphorylated also by deoxycytidine kinase (see Cozzarelli, 1977) and the phosphorylation of arabinosyl-cytosine is a prerequisite for its cytostatic effect (Cohen, 1966; Cozzarelli, 1977). In this respect, our data indicate an apparent discrepancy, i.e. deoxycytidine kinase shows a lower activity in the intact cells, but the lymphocytes are far more sensitive to arabinosyl-cytosine inhibition than the purified enzyme (Figs 1, 4, 5). However, this problem may be solved by taking into account the facts, that arabinosyl-cytosine is in competition with deoxycytidine (Fig. 5). At the inhibition of the purified enzyme, deoxycytidine concentrations were near to saturation (Fig. 5), while in the cells there are much lower concentrations of unphosphorylated deoxynucleosides (Plagemann et al., 1978). Recently it has been suggested by Mejer (1982) that the administration of arabinosyl-cytosine together with deoxycytidine could protect normal cells more than malignant ones. This proposal was based on the finding that the ratio of arabinosyl-cytosine to deoxycytidine phosphorylation is twice as large in leukemic cells as in normal pheripheral lymphocytes. These and other data (Chang et al., 1982; Hershfield et al., 1982; Cheng et al., 1977) raised the possibility that there are more deoxycytidine kinase isoenzymes different in their properties. Therefore, rapid methods of purification are desirable to obtain enzymes suitable for molecular and kinetic characterization.

In this paper we applied the same purification for deoxycytidine kinase as used previously for separation of thymidine kinase isoenzymes from human tonsillar lymphocytes (Sasvári-Székely et al., 1985). The deoxycytidine kinase was bound at low ion strength and pH: 7, and it was eluted by a linear gradient of KCl (see Fig. 2), resulting in a 38–40-fold purification. About the same extent of purification was obtained by affinity chromatography on Cibachron Blue 3G-A for deoxycytidine kinase from human leukemic lymphocytes (Baxter et al., 1978).

Some properties of the partially purified human tonsillar deoxycytidine kinase are similar to the thymic enzyme, i.e. its activity decreased during storage, which could be restored by preincubation with dithiothreitol (Ives and Durham, 1970). The tonsillar enzyme was very sensitive to dCTP inhibition, while the other deoxyribonucleoside triphosphates had no effect in the same concentration range (Durham and Ives, 1970). It has a rather low molecular weight, which is also similar to deoxycytidine kinase from other tissues (Ives and Durham, 1970; Momparler and Fischer, 1968; Kessel, 1968). With deoxycytidine as substrate, regular Michealis-Menten kinetics were obtained (Fig. 5) with an apparent K_m value of 13 μM,

similar to the thymus enzyme (Momparler and Fischer, 1968) and leukemic cells (Kessel, 1968). However, bimodal double-reciprocal plots were shown in some laboratories (Durham and Ives, 1970; Kozal et al., 1972), giving a lower (5 μM) and a higher (50 μM) apparent K_m value. Deoxycytidine kinase also phosphorylates a series of other nucleosides, but with considerably higher apparent K_m values (for rew. see Anderson, 1973), and all available evidence indicates that they compete with deoxycytidine for the same enzymic site (Ives and Durham, 1970; Momparler and Fischer, 1968). Our finding that arabinosyl-cytosine exhibits a competitive inhibition with deoxycytidine is in good agreement with these data.

The controversy between the lower in vivo incorporation and higher in vitro phosphorylation of deoxycytidine compared with deoxythymidine may lie at the level of nucleoside interconversion mechanisms, which seems to be rather characteristic of the state of cell differentiation than of cell proliferation.

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Stability, Heat Stability and Heat Sensitivity of Proteins: Thermodynamic Considerations

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The heat sensitivity and stability of a protein should be characterized by the Arrhenius parameters of the irreversible denaturation. The terms "thermostability" or "activation enthalpy" and "activation entropy" of denaturation as "thermodynamic parameters of protein stability" are misleading. The terms "measure of heat sensitivity" and "measure of stability" should be used as comparative data for protein structure.

For many years various enzymes have been studied extensively in order to elucidate the mechanism underlying the exceptional thermostability of proteins isolated from thermophilic bacteria (Singleton and Amelunxen, 1973; Zuber, 1976). In these and other studies the terms "heat stability" and "stability" of proteins are used as synonyms thus creating some confusion. We are going to discuss below whether the two features of the protein molecule are identical or not, and will suggest the introduction of the term "heat sensitivity". (This term is equivalent with the thermotolerance of Fujita 1978.)

It has long been realized that during protein denaturation two separate steps must be distinguished: *(i)* reversible conformational change or unfolding, and *(ii)* irreversible conformational change followed by aggregation and coagulation of the unfolded product:

$$N \rightleftharpoons D_R \longrightarrow D_I \longrightarrow P$$

where N stands for the native protein, D_R and D_I for the reversibly and irreversibly denatured protein, respectively, and P for precipitated protein. Each species is assumed to be a population of protein molecules with more or less different conformations. In the following the first step will be called reversible denaturation, the total process is precipitation and the process up to the first irreversible step will be referred to as irreversible denaturation. The $D_R \rightarrow D_I$ transition is probably a formal description of a number of parallel irreversible reactions. These, however, are frequently characterized by a single resultant first order rate constant.

While thermal denaturation studies are usually carried out on irreversible denaturation processes, with some proteins reversible denaturation can be achieved. The theoretical considerations on protein stability and denaturation are based exclusively on reversible denaturation processes (Scheraga, 1961, 1963; Joly, 1965; Tanford, 1968, 1970; Ackermann, 1969; Poland and Scheraga, 1970; Lauffer, 1975; Edelhoch and Osborne, 1976; Sturtevant, 1977; Biltonen and Freire, 1978; Pfeil and Privalov, 1979). Privalov and co-workers have demonstrated by calorimetric

measurements that the assumption according to which only two states (N and D_R) are involved in the reversible denaturation processes is a very reasonable approximation for five small proteins (Pfeil and Privalov, 1979), or it is true for the single domains of bigger proteins (Privalov, 1982). However, the study of thermodynamics of the reversible denaturation process characterizes only an equilibrium under the given conditions. The Arrhenius parameters of the irreversible process characterize the heat sensitivity (Fujita, 1978; Fujita and Imahori, 1974; Fujita et al., 1979) or the stability of a protein (cf. Simon, 1981) in a more informative manner since reflect the energy needed for the disruption of bonds responsible for the maintaining the native structure. Therefore we suggest that for the definition of stability and heat sensitivity of proteins the irreversible denaturation ($N \rightarrow D_I$) should be considered instead of reversible denaturation. Experimentally this means the use of the Arrhenius parameters instead of the van't Hoff parameters, but the former should not be confused with the so-called "kinetic stability" which is an apparent stability due to the small kinetic constant of protein unfolding as compared to the time of experiment (Lumry and Biltonen, 1969).

The rate constant or its equivalent, the activation free energy change of irreversible denaturation at a given temperature is assigned to characterize the stability of the proteins.* The stability of a protein refers to a given temperature, but if the change in the so-called "activation entropy" multiplied by the absolute temperature is nearly equal with the so called "activation enthalpy" at various temperatures, the stability of the protein will not change (compensation effect,** cf. Lumry and Rajender, 1970).

The heat sensitivity is assigned to be characterized by the "enthalpy of activation", ΔH^*. The transition of native protein molecules to irreversibly denatured ones (the $N \rightarrow D_I$ process) requires a well-defined "activation energy" which is proportional to the energy requirement of formation of false folding nuclei (Simon, 1981). To obtain the equivalent thermal energy, a given temperature is needed.

The greater is ΔH^* the more pronounced is the temperature dependence of the irreversible denaturation, i.e. the protein is more heat sensitive. If ΔH^* depends on temperature, i.e. the change of heat capacity, ΔC_p, differs from zero – and this is generally the case (Tanford, 1968, 1970) – then instead of ΔH^* both ΔH^* and ΔC_p should be considered.

The heat sensivity, being characterized by the change in "activation enthalpy" and heat capacity of protein unfolding (i.e. the energy barrier needed for the transition from N to D_I), differs from stability in the "entropy" factor which charac-

* This stability should be similar for the majority of proteins of the mesophilic organisms. In fact the ΔG^* of irreversible denaturation of proteins (near the physiological temperature) is around 100 kJ/mole, independently of the nature of the protein (Joly, 1965).

** However, it is to be noted that the observed distribution of data points along straight lines in the "enthalpy-entropy" plane is more often due to the propagation of measurement errors than to chemical variations, i.e. the compensation effect is often an artefact (Banks et al., 1972; Krug et al., 1976, 1976a, 1976b)

terizes the degree of order (i.e. the number and strength of H-bonds, van der Waals forces, ionic bonds, etc.) of the transition state from native to denatured form.

Two proteins which exhibit different heat sensitivities may have identical stabilities and *vice versa*. However, it is meaningless to speak generally of the "thermostability" of a protein, since if a protein is more "thermostable" than another in a given temperature range, in another temperature range it may be less "thermostable" (Fujita, 1978; Váli et al., 1980). The term "thermostability" is meaningful only in comparison with other proteins in a given temperature range. In contrast, the stability at a given temperature or the heat sensitivity of a protein is its inherent and invariable property if strictly controlled conditions are assured.

Moreover, one should be aware that the Arrhenius equation, originally, refers to a single elementary step. The irreversible denaturation of a protein is never the result of a single elementary step, but of the sum of hundred or thousand partially consecutive, partially parallel elementary steps of the motion of different side chains, rupture of H-bonds, van der Waals forces, hydrophobic interactions, etc. Consequently, the terms of "activation enthalpy", "activation entropy", "activation energy" or "activation free energy" of protein denaturation are misleading and not recommended (cf. Keleti, 1983). We suggest to call the "enthalpy of activation (ΔH^*) of heat denaturation" as *measure of heat sensivity* and the "activation free energy (ΔG^*) of heat denaturation" as *measure of stability*. This may help to avoid the confusion arising from the fact that some peoples think that these very useful comparative data are real "thermodynamic features of protein thermostability".

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Regulation of beta-adrenergic Sensitivity by Guanine Nucleotides in Rat Brain

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Binding of β-adrenergic receptor by labelled dihydroalprenolol was inhibited in the presence of GTP or GppNHp in rat brain particulate fractions. The inhibition was suspended when the particulate fraction was pretreated at 30 °C for 20 minutes and washed 3 times. It is concluded that the inhibition was due to endogenous bound catecholamines.

Investigations of the last ten years proved that beside beta-adrenergic receptor and adenylate cyclase a third factor, the G/F or N protein has an important role not only in the receptor-cyclase coupling but also in the sensitization and desensitization of beta-adrenergic receptor (Ross et al., 1978; Cassel and Selinger, 1978).

Desensitization or sensitization of receptors are caused most frequently by high resp. low hormone levels in vivo (Axelrod, 1974). The chronic administration of certain drugs, changes of ion and nucleotide concentrations, or variations in the synthesis or degradation of protein and lipid components may also produce desensitization (for review see Wollemann, 1978).

Our own investigations were carried out with in vitro systems since 1975 (Wollemann and Rózsa), when we published that serotonin activates more effectively the mussel heart adenylate cyclase and catecholamines the rat heart adenylate cyclase, provided that they were previously in vivo treated with reserpine, i.e. the endogenous level of serotonin and catecholamines were depleted (Pik and Wollemann, 1977). When the hormone action was investigated in details in rabbit heart membrane fractions an increase in the number of beta-adrenergic receptors was established and an enhancement of the isoproterenol activation on the adenylate cyclase activity was found after reserpine treatment. At the same time the basal activity of adenylate cyclase, which was measured in the presence of guanine nucleotides, decreased substantially in the reserpine treated heart membranes (Tkachuk and Wollemann, 1979).

The aim of our present experiments was to demonstrate the role of guanine nucleotides on the binding of beta-adrenergic receptors by labelled dihydroalprenolol. It was known from earlier data, that guanine nucleotides shift the competition curves for agonists to the right (Maguire et al., 1976 and Lefkowitz et al.

Abbreviations: DHA, dihydroalprenolol; GTP, guanosine triphosphate; GppNHp, 5'-guanylyl-imidophosphate

1976). From our previous investigations on rabbit heart (Tkachuk and Wollemann, 1979) and recently from guinea pig brain (Yamamoto and Shimizu, 1983) it was demonstrated, that guanine nucleotides have a dual regulatory role on adenylate cyclase activity, which means that stimulatory as well as inhibitory actions were found. Interestingly the adenylate cyclase inhibited by guanine nucleotides was more sensitive to hormonal stimulation. Therefore the performing of similar experiments on the action of guanine nucleotides on the beta-adrenergic receptor in brain was our next goal.

Materials and methods

(−)Alprenolol hydrochloride was purchased from Hässle, propranolol from ICI, all other fine chemicals were obtained from Sigma. (^{3}H)dihydroalprenolol (65 Ci/mmol) was prepared as described (Borsodi et al., 1983).

PVG/C rats were killed by decapitation the brain was removed and homogenates were prepared with 9 vol. 75 mM pH 7.5 Tris-HCl buffer containing 25 mM $MgCl_2$ using a Potter homogenizer. The homogenate was centrifuged and washed three times with the above buffer as described previously (Horváth et al., 1979). Part of the experiments were performed after preincubating the resuspended pellet at 37 °C for 20 min in order to remove endogenous ligands.

Binding assay was carried out in triplicates in a volume of 250 μl containing 100–200 μg protein, 75 mM pH 7.5 Tris-HCl puffer, 25 mM $MgCl_2$ and various amounts of labelled dihydroalprenolol. Samples were incubated at 0 °C for 5 min if not otherwise indicated. Nonspecific binding was measured in the presence of 10 μM propranolol, according the method of Lefkowitz et al. (1978). Samples were filtered under vacuum on Whatman GF/C glass fiber filters and washed twice with 10 ml of the above described buffer solution. The radioactivity of the dried filters was counted by liquid scintillation spectrometry in toluene-based cocktail. Specific binding was linear within the used protein concentration range. Protein content was determined according to Peterson (1977).

Results and discussion

Investigating the time course of GTP and GppNHp effect on beta-adrenergic receptor binding using labelled dihydroalprenolol and inhibitory effect was observed with both substances, GppNHp being about ten times more effective than GTP (Figs 1 and 2).

Varying the concentrations of labelled dihydroalprenolol GppNHp reduced the number of binding sites of the beta-adrenergic receptor (Fig. 3). However preincubation of the particulate fraction at 30 °C for 20 minutes abolishes most of the GppNHp inhibitory effect (Fig. 4) in addition above the saturation level of specific binding (20 nM) GppNHp increases the exchangeable, but not specific binding.

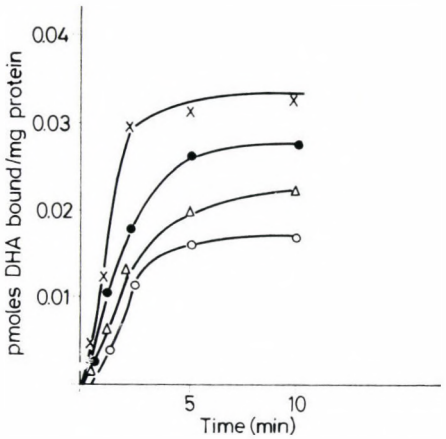

Fig. 1. Effect of GTP on the time course of 10 nM labelled DHA specific binding of β-adrenergic receptor from rat brain particulate preparation. $\times-\times$ control; $\circ-\circ$ 10^{-5} M GTP; $\triangle-\triangle$ 10^{-4} M GTP; $\bullet-\bullet$ 10^{-3} M GTP. Points represent the mean value of triplicate samples

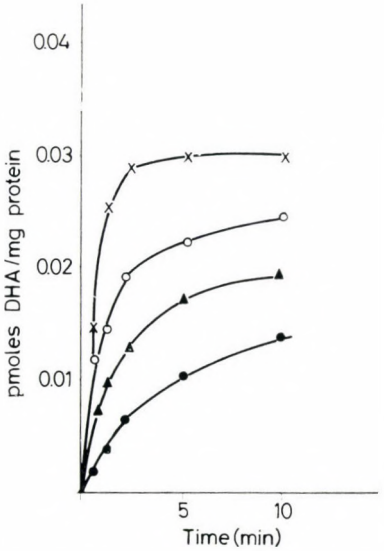

Fig. 2. Effect of GppNHp on the time course of 10 nM labelled DHA specific binding of β-adrenergic receptor from rat brain particulate preparation. $\times-\times$ control; $\bullet-\bullet$ 10^{-4} M GppNHp; $\triangle-\triangle$ 10^{-5} M GppNHp; $\circ-\circ$ 10^{-6} M GppNHp. Points represent the mean value of triplicate samples

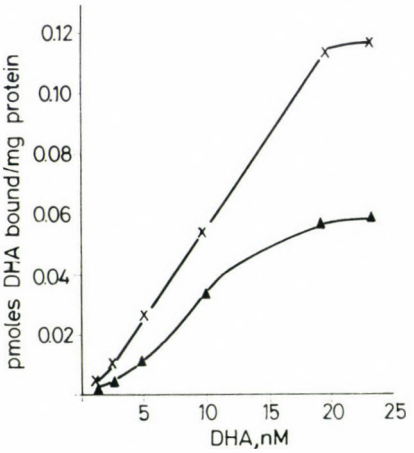

Fig. 3. The effect of GppNHp on different concentrations of labelled DHA specific binding of the β-adrenergic receptor from rat brain particulate preparation. $\times-\times$ control, $\triangle-\triangle$ GppNHp 10^{-4} M. Points represent the mean value of triplicate samples

Therefore, it is concluded that the GppNHp inhibition of labelled DHA binding might be affected by the remaining, firmly bound catecholamines in the particulate preparation, which are metabolized at higher temperature. Whether this inhibitory effect is exerted through the N_i or N_s (inhibitory or stimulatory nucleotide regulating protein), (Hildebrandt et al., 1983) remains to be established.

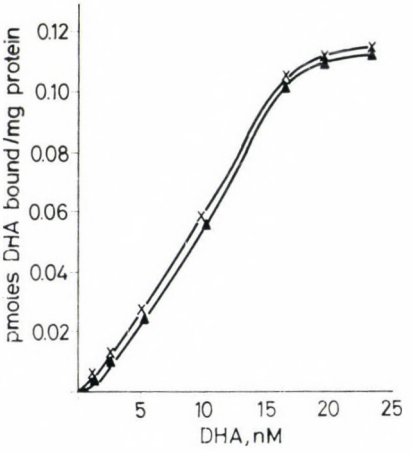

Fig. 4. The effect of GppNHp on different concentrations of labelled DHA specific binding of the β-adrenergic receptor from rat brain particulate preparation pretreated at 30 °C for 20 minutes. $\times-\times$ control; $\triangle-\triangle$ GppNHp 10^{-4} M. Points represent the mean value of triplicate samples

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Analysis of Ca^{2+}-induced K^+-transport of Human Erythrocytes in Propionate Media

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A method is described which allows studying specific cation transport pathways of the cell membranes by converting ion fluxes into volume changes. Lipophylic weak electrolytes, such as propionate, rapidly penetrate the cell membranes in their undissociated acid from but not as negatively charged ions. When human red cells are incubated in isoosmotic K-propionate media an intracellular acidification occurs with a limited propionate uptake and volume increase (corresponding to the buffering capacity of the cytoplasm). If both protons and alkali cations are rendered permeable, a rapid salt influx and volume increase is observed. The latter can be quantitatively followed by electronic sizing methods. A detailed characterization of the system is provided through studies with ionophores, inhibitors of the red cell anion exchange system and drugs which activate or inhibit the Ca^{2+}-induced K^+ transport. It is demonstrated that in K-propionate media the permeability of the Ca^{2+}-induced K^+ pathway can be directly estimated. The method is suitable to observe population (all-or-none) responses in the activation of the K^+ pathway under certain experimental conditions. The application of the method in the search for cation-proton exchanger systems is discussed and its use for demonstration purposes by producing selective lysis of red cells is described.

Introduction

Ca^{2+}-induced K^+ transport in human red cells (Gárdos, 1956, 1958) and in various non-excitable and excitable cells (for reviews see Lew and Ferreira, 1978; Mech, 1978; Lew and Beaugé, 1979; Schwarz and Passow, 1983; Sarkadi and Gárdos, 1985) is a widely investigated system with a probable basic physiological significance in triggering metabolic or secretory responses and modifying the excitability of several tissues. The effect is a selective, rapid increase in the K^+ permeability of the cell membrane induced by the interaction of Ca^{2+} with the intracellular membrane surface (Lew, 1970; Blum and Hoffman, 1972). The role of Ca^{2+}, the effects of various ions on the triggering event, as well as the changes in membrane potential produced by the opening of the conductive K^+ pathway, are well characterized in the literature (see reviews above). The involvement of cellular metabolic intermediates and Ca^{2+}-dependent proteins such as calmodulin in evoking or modulating K^+ transport have been thoroughly investigated but conclusive evidence are still not available in these respects. An all-or-none characteristic of the opening of the K^+ pathway has been indicated and its possible causes debated (Hoffman et al., 1980; Simonsen et al., 1982; Garcia-Sancho et al., 1982; Lew et al., 1982).

In the present paper we describe a method which is suitable to investigate cation transport processes by the application of a weak electrolyte, propionate. The use of this method allows the conversion of ion movements into volume changes which can be easily followed by techniques such as light scattering, direct photometry, or electronic sizing. The use of light scattering for volume measurements as simple means to distinguish transport pathways has been advocated by Hladky and Rink (1982). Here we used the electronic sizing (Coulter Counter) because under the conditions applied this latter system, in contrast to the light scattering method, is insensitive to changes in red cell shape and plasticity. We also demonstrate that the technique can be used for demonstration purposes without any expensive instrumentation, by following selective hemolysis.

Materials and methods

Gramicidin, nigericin, CCCP (carbonilcyanide m-chlorophenylhydrazone), SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid), quinine and propionic acid were purchased from SIGMA Chemical Co. Valinomycin and A23187 were obtained from Calbiochem, propranolol from SIGMA and RPMI incubation medium from Gibco Labs.

Isoosmotic K-propionate solutions contained 140 mM K-propionate, 20 mM Tris-Cl (pH 7.0), 2 mM $MgCl_2$, 1 mM $CaCl_2$ (or when indicated 1 mM EGTA), and 10 mM glucose. Freshly drawn human red cells were washed 3 times with RPMI medium. Cell volume was measured by placing 2×10^5 cells in 20 ml of buffer solutions and using the Coulter Counter–Channelyzer system, as described by Grinstein et al., 1982. The diameter of the aperture of the electrode used was 100 μm to assure that changes in red cell shape would not cause an apparent volume change. This was tested by adding 1 μM A23187 + 1 mM Ca^{2+} to the cells in isoosmotic media containing 120 mM KCl, 20 mM NaCl and 20 mM Tris-Cl, pH 7.0. The immediate changes in red cell shape under this condition (formation of spherocytes) did not produce any apparent volume change.

Intracellular pH was estimated by the lysis of packed red cells in 10 fold volumes of distilled water, freezing and thawing the cells to assure complete hemolysis and measuring pH by a combination glass electrode.

Results and discussion

In experiments such as presented in Figs 1 and 2, we examined the effects of propionate media and various ionophores on the volume changes in human red cells. If red cells were incubated in an isoosmotic K-propionate medium (pH 7.0) there was no significant volume change observable. The intracellular pH in such cells was found to be 6.95, only slightly lower than that of the medium. If red cells were pretreated with 100 μM SITS in a NaCl medium and then placed in K-pro-

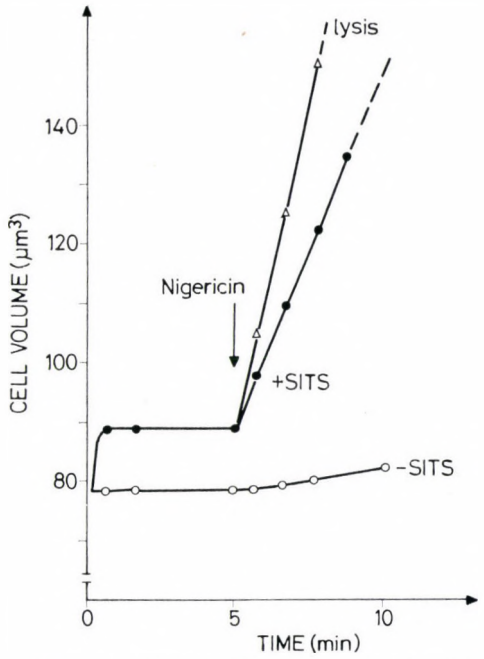

Fig. 1. Volume changes of human red cells in K-propionate media. E fects of SITS and nigericin. ○ control cells; ● +100 μM SITS. At the time indicated by the arrow 1 μM (●) or 2 μM (△) nigericin was added to the media

pionate media (or if this latter medium contained similar SITS concentrations) a rapid swelling was observed, yielding a 12–15% volume increase within 30 seconds. No further volume change was obtained within 15–20 minutes. Intracellular pH of the cells incubated in K-propionate media with SITS was found to be between 6.5–6.55. Addition of the ionophore nigericin, carrying an obligatory exchange of Na^+, K^+, or H^+ (see Pressman, 1976), produces no significant changes in the red cell volume if added to the control cells after 5 min in K-propionate media (Fig. 1). In contrast, nigericin in the SITS-treated cells leads to a rapid volume increase and eventual lysis of the cells. Increasing nigericin concentrations between 0.1 to 2 μM further increase the rate of swelling. If the ionophore is added to the control (no SITS) cells at the time of placing them in K-propionate, a swelling response is also obtained, although less rapid and pronounced than in the SITS-treated cells.

The probable explanation of the above findings is that undissociated propionic acid is lipid-soluble and rapidly penetrates the cell membrane, while propionate$^-$ has a very limited permeability (Keifer, 1981; deHemptinne et al., 1983). Propionic acid rapidly distributes between the medium and the cytoplasm and dissociates again in the cell interior to propionate$^-$ and H^+. Human red cells possess a high capacity anion exchanger system which compensates this increase in intracellular H^+ concentration rapidly. Cellular Cl^- is exchanged for external HCO_2^-

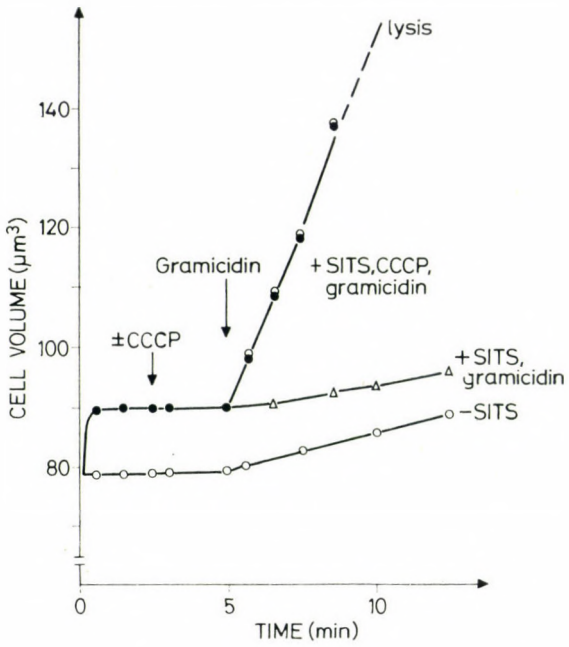

Fig. 2. Volume changes of human red cells in K-propionate media. Effects of SITS, CCCP and gramicidin. ○ control cells; ● +100 μM SITS. At the arrows CCCP (10 μM) or gramicidin (0.5 ●, or 1 μM ∅, ▲) was added to the media

which recombines with the protons and dissociates into H_2O plus CO_2. These final products are permeable through the the cell membrane thus the result is an exchange of cellular Cl^- for external propionate without significant pH changes (see Appendix, condition A). If rapid anion exchange is blocked by a specific inhibitor such as SITS (see Knauf, 1979), pH equilibration cannot take place but a certain amount of H^+ is buffered by intracellular proteins, in red cells mostly by hemoglobin. This buffering produces a limited propionate$^-$ uptake and cell swelling. This is a rapid process (completed within seconds) and leads to the stabilization of the cell volume at a slightly higher value and of the cellular pH in the acidic range (Appendix, condition B). The cellular pH measured in these experiments indicates an intracellular propionate$^-$: propionic acid ratio of about 60 (propionic acid is supposed to distribute uniformly between the cells and the medium), thus about 40 mM H^+ had to be buffered during a pH decrease of 0.5 unit. This value corresponds to the reported buffering capacity of hemoglobin, about 60–65 mmoles/litre of cell/pH unit (Wyman, 1948). The observed volume increase of 12–13% also corresponds to the amount of the accumulated osmotically active propionate$^-$. If a cation-proton exchanger ionophore is added to the cells incubated in

isoosmotic propionate media (the anion exchanger is blocked), H^+ can leave the cells in an exchange for external cations. This process leads to a net cation + propionate$^-$ uptake, osmotically obliged water influx and a cell swelling (Appendix, condition C). The process eventually results in hemolysis.

Figure 2 illustrates experiments in which red cells were incubated in K-propionate media and treated with the protonophore CCCP or the cation-channel forming antibiotic, gramicidin. As seen, these compounds, when added separately, do not produce significant swelling whether SITS is present or not. However, in SITS-treated red cells the combination of the two ionophores leads to a rapid swelling and hemolysis. Gramicidin at near neutral pH does not carry significant H^+ flux (Hladky and Rink, 1982), thus a cation $-H^+$ exchange cannot take place. CCCP has no effect either, since the requirement for electroneutrality does not allow H^+ efflux without a concomittant cation influx. The combination of CCCP and gramicidin functions as a cation − proton exchanger producing similar effects as nigericin. At a constant CCCP concentration (10 μM) increasing the gramicidin concentration from 0.5 to 2 μM does not modify the rate of swelling, while altering the CCCP concentration between 1–10 μM at a constant (1 μM) gramicidin concentration significantly facilitates the process. Thus the rate-limiting factor under these conditions is the H^+ flux catalyzed by the mobile carrier CCCP and not the cation flux allowed by the high-capacity channel forming gramicidin. These experiments provide additional information on the low intrinsic H^+ and OH^- permeabilities of the red cell membrane.

The system such as characterized be these ionophore studies can be used for the investigation of natural membrane transport pathways such as the Ca^{2+}-induced K^+ transport. Increase of the cellular Ca^{2+} concentration by using the divalent cation ionophore A23187 is a well known way to evoke rapid K^+ transport (Reed, 1973; Reed, 1976; Gárdos et al., 1975; Ferreira and Lew, 1976). Figure 3 demonstrates the effect of A23187 + Ca^{2+} or EGTA on the volume changes in human red cells incubated in K-propionate media. The cells were pretreated with SITS in order to block the anion exchange pathway. The addition of A23187 did not produce any significant volume change either in the presence or absence of Ca^{2+} if CCCP was absent. However, in the presence of the protonophore CCCP, A23187 + Ca^{2+} induced a rapid volume increase and eventual cell lysis. The phenomenon was blocked by the removal of external Ca^{2+} (by the addition of EGTA) or by the inhibitor of the Ca^{2+}-induced K^+ transport, quinine (Armando-Hardy et al., 1975; Reichstein and Rothstein, 1981). Neither EGTA nor quinine had any effect on the volume changes produced by nigericin or the combination of CCCP + + gramicidin. Quinine is also known not to affect the Ca^{2+} movement catalyzed by A23187 (Ferreira and Lew, 1976). The need for the presence of CCCP to see volume changes indicates that the Ca^{2+}-induced K^+ transport pathway activated by A23187 + Ca^{2+} carries a net K^+ transport but (at the pH values examined) no K^+-H^+ exchange. The ionophore A23187 may carry a certain amount of proton flux in exchange for divalent cations but its magnitude is apparently not enough to contribute to the volume changes.

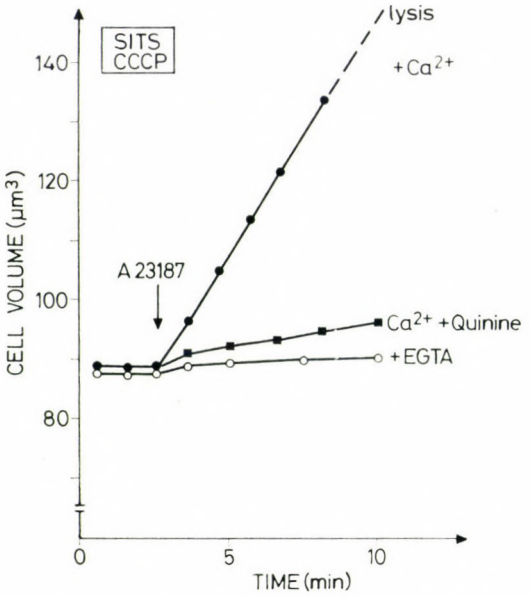

Fig. 3. Effects of A23187 ionophore on the red cell volume in K-propionate media. Red cells were incubated in isoosmotic K-propionate media containing 100 μM SITS and 10 μM CCCP. A23187 ionophore was added at the time indicated by the arrow. ● 1 mM Ca^{2+}; ■ 1 mM Ca^{2+} + 200 μM quinine; ○ 1 mM EGTA

In the presence of 10 μM CCCP and the maximum effective A23187 + Ca^{2+} concentrations (A23187 = 0.5–2 μM; Ca^{2+} = 1–5 mM) the initial rate of swelling (7–8 μm^3/min) does not reach the rate of of the gramicidin-induced response (12–15 μm^3/min), indicating that the concentration of CCCP is not rate-limiting. Thus the rate of swelling probably directly reflects the conductivity of the Ca^{2+}-induced K^+ channels. From these data the maximum conductive K^+ flux through this pathway is estimated to be about 12–15 mmoles/litre of cell/min.

Using A23187 concentrations of 1 μM and altering extracellular Ca^{2+} concentrations between 0.1 μM to 1 mM in a Ca-EGTA buffer, the swelling response is maximum above a free Ca^{2+} concentration of 1 μM (data not shown). Thus the system shows a "high Ca^{2+}-affinity" pattern, as reported by Lew and Ferreira (1976). At lower A23187 concentrations (0.01–0.2 μM) the response becomes variable and requires higher Ca^{2+} concentrations (above 0.5 mM) to be observed. A partial (population) response of the cells is also observed (see below). At such low ionophore concentrations the binding of A23187 to the cell surface or to the plastic vials makes these determinations unreliable.

The addition of propranolol + Ca^{2+} to human red cells is another established method to induce Ca^{2+}-dependent K^+ transport (Ekman et al., 1969; Mannninen, 1970). The mechanism of triggering is not entirely known but its probable basis is a transient increase in cellular Ca^{2+} and an increased sensitivity of the cell

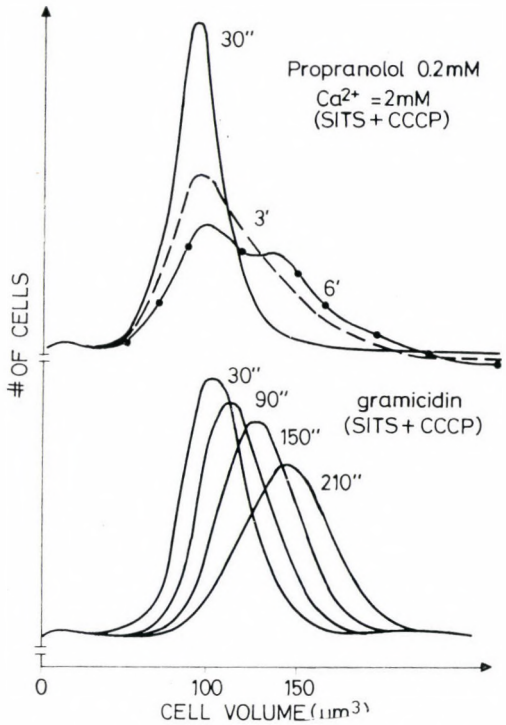

Fig. 4. Population (all-or-none type) response in the volume changes of red cells incubated in K-propionate media. The isoosmotic K-propionate media contained 100 μM SITS and 10 μM CCCP. The measurements were performed at the times indicated on the figure. Upper part: Effect of 0.2 mM propranolol + 2 mM Ca^{2+}; Lower part: Effect of 0.5 μM gramicidin

membrane to Ca^{2+} (Porzig, 1975; Szász et al., 1977). Red cell swelling in a K-propionate medium can also be evoked by propranolol + Ca^{2+}, provided that SITS and CCCP are present. The maximum response, which is similar to that produced by A23187 + Ca^{2+}, is observed at 0.2–0.3 mM propranolol and 10 mM Ca^{2+}. This phenomenon is also inhibited by 100 μM quinine (data not shown). In the presence of propranolol concentrations below 0.2 mM or above 0.5 mM and at Ca^{2+} concentrations below 10 mM the response is partial and an all-or-none type effect is observed. As shown in Fig. 4, at 0.2 mM propranolol and 2 mM Ca^{2+}, some of the red cells rapidly swell, while others retain their original volumes. Under the given conditions, after about 6 minutes of exposure to propranolol + Ca^{2+}, the populations of responding and non-responding cells clearly separate on the Coulter graph. After this time a hemolysis of the swelling cells obscures the picture as the lysed cells rapidly regain their initial (or slightly smaller) volumes. As a contrast to this all-or-none type of response, on Fig. 4 we also demonstrate the Coulter graphs obtained in the presence of CCCP + gramicidin.

Fig. 5. Hemolysis of red cells in K-propionate media. Washed red cells with a final hematocrit of 0.5% were incubated in 0.7× isoosmotic K-propionate media containing 100 μM SITS. The photograph was taken 10 minutes after the addition of the following compounds: Tube 1: none; 2: 2 μM nigericin; 3: 10 μM CCCP; 4: 1 μM gramicidin; 5: 10 μM CCCP + 1 μM gramicidin; 6: 1 μM A23187 + 1 mM Ca^{2+}; 7: 10 μM CCCP + 1 μM A23187 + 1 mM Ca^{2+}; 8: 10 μM CCCP + 1 μM A23187 + 1 mM EGTA

All the above experiments indicate that the propionate method can be applied to investigate the Ca^{2+}-induced K^+ transport in red cells by converting cation fluxes into volume changes. The direct demonstration of a heterogeneous response is a significant advantage of the method.

In the field of membrane biology the demonstration of transport phenomena often requires complicated instrumentation not available in high school or university laboratories. The propionate method allows such a classroom demonstration based on the selective lysis of red cells. The demonstration is made easier if the red cells are incubated in slightly hypoosmotic K-propionate media as the threshold for hemolysis is decreased under these conditions. Figure 5 illustrates such a demonstration based on hemolysis in K-propionate media. In this particular case red cells with a hematocrit of 0.5% were suspended in 0.7× isoosmotic K-propionate media containing 100 μM SITS. The ionophores and other ingradients were added after the cells were suspended in the medium. The photograph, taken 10 minutes after the addition of the compounds indicated in the legend to the figure, shows the imminent hemolysis in the tubes where a cation $-H^+$ exchange took place. The use of a simple photometer to check for the sudden change in the absorbance or light scattering at the time of the hemolysis allows quantitative studies as the hemolysis time can be correlated to the rate of cell swelling.

In the present paper we demonstrated the applicability of the propionate method to analyze the Ca^{2+}-induced K^+ transport in red cells. The method is also suitable to follow other cation transport processes if they allow substantial ion movements producing measurable volume changes. The method has been successfully applied to study $Na^+ - H^+$ exchange in lymphocytes (Grinstein et al., 1984) and in chinese hamster ovary cells (Sarkadi et al., 1984), since intracellular acidification activates such a pathway.

Appendix

The reactions underlying the phenomena described in this communication when red cells are placed into isoosmotic propionate media can be summarized by the following equations:

It is assumed that water is rapidly permeable through the cell membrane and the cells behave as osmometers. Index $_0$ means outside, $_i$ intracellular components.

Condition A: the membrane is permeable for undissociated propionic acid but is not conductive for propionate$^-$, alkali cations, H^+ and OH. The membrane possesses of a rapid anion exchanger system.

1. $\text{propionate}_0^- + H_0^+ \rightleftharpoons \text{propionic acid}_0$; (pK = 4.75)
2. $\text{propionic acid}_0 \rightleftharpoons \text{propionic acid}_i$;
3. $\text{propionic acid}_i \rightleftharpoons \text{propionate}_i^- + H_i^+$;
4. $Cl_i^- + HCO_{3\,0}^- \rightleftharpoons Cl_0^- + HCO_{3i}^-$;
5. $HCO_{3i}^- + H_i^+ \rightleftharpoons H_2CO_{3i} \rightleftharpoons H_2O + CO_2$

The amount of CO_2 dissolved in a medium equilibrated with air at pH 7.0 produces about 100 μM of HCO_3^-, sufficient to allow rapid pH equilibration through the anion exchanger. The net result is an exchange of internal Cl^- for external propionate$^-$.

Condition B: the membrane is permeable for undissociated propionic acid but not for anions or cations.

1–3: as in A, obtaining $\text{propionate}_i^- + H_i^+$;

4. $\text{Hemoglobin}_i^{X-} + H_i^+ \rightleftharpoons \text{Hemoglobin-H}_i$

A certain amount of H^+ is buffered by cellular hemoglobin and the uptake of propionate$^-$ and the corresponding volume increase are determined by this buffering capacity. Hemoglobin buffering of protons also occurs in condition A, but the rapid anion exchange makes this effect negligible.

Condition C: the membrane is permeable for undissociated propionic acid but not for cations or anions. A specific cation$-H^+$ exchanger is added to the membrane.

1–3: as in A, obtaining $\text{propionate}_i^- + H_i^+$;

4. $\text{cation}_0 + H_i^+ \rightleftharpoons \text{cation}_i + H_0^+$.

The uptake of cation + propionate$^-$ leads to a cell swelling.

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Liposome Mediated DNA-transfer into Mammalian Cells

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We have investigated the interaction of mammalian cells with liposome encapsulated DNA. Tissue cultured mammalian cells were exposed to large, unilamellar phosphatidyl serine liposomes containing DNA molecules from different animal cells or prokaryotic organisms.

The liposomes bind rapidly to the surface and are taken up by the cells and significant proportion of the encapsulated DNA is transported to the nuclei.

Transient expression of the foreign genetic material could be detected in high percentage of the treated cells for a few days. During this period of time foreign DNA is present in both free and integrated form, however, the free form soon disappears.

Stable transformant cell colonies— with continuous expression of new gene(s)— were isolated under selective pressure with a frequency of approx. 10^{-5}.

Several techniques have been developed for the transfer of foreign DNA sequences into eukaryotic cells or protoplasts (McBride and Ozer, 1973; Pellicer et al., 1980; Graham and van der Eb, 1973; Fraley et al., 1980). One of the most promising methods uses artificial lipid vesicles (liposomes), providing protection against nucleases and delivering the DNA molecules into the cytoplasma by endocytosis or fusion with cellular plasmamembranes.

In this paper we report details of cell-liposome interaction, and the fate of foreign DNA sequences in the target cells. Furthermore, expression and physical state of the microinjected DNA will be discussed.

Materials and methods

Cell culture

Rat H56 tk^- cells (kindly provided by Dr. M. Weiss) and CHO-Kl pro^- chinese hamster ovary cells were maintained in F12 medium containing 5% calf serum. Mouse SL tk^- cells (obtained from Dr A. Venetianer) were grown in MEM supplemented with 5% calf serum.

Abbreviations: MEM: minimal essential medium; LUV: large, unilamellar vesicles; PBS: phosphate buffered saline; SSTKNa buffer: 0.34 M sucrose, 0.15 mM sperine, 0.5 mM spermidine, 15 mM tris (pH 8.0), 60 mM KCl, 15 mM NaCl; TCA: trichloroacetic acid; HAT medium: tissue culture medium, containing hypoxanthine, adenine and thymine; HSV-TK: thymidine kinase gene sequence of Herpes Simplex Virus.

DNA

DNA was purified from *E. coli*, lambda phage, cultured drosophila cells (KC cells), chick embrios, human embrionic liver of chicken red blood cells according to standard procedures.

Labelled DNA was purified from E. coli cells grown on a phosphate free medium supplemented with 5 mCi (200 MBq) ^{32}P orthophosphate. In vitro labelling of lambda phage particles or lambda DNA with 125, I was carried out according to Hunter and Greenwood (1971) and Commerford (1971), respectively.

Liposomes

Large unilamellar phosphatidyl serine vesicles were prepared according to Papahadjopoulos et al. (1975), purified by 1 M NaCl precipitation. Treatment of cells with liposomes was carried out as described earlier.

Uptake of liposome entrapped, labelled DNA by cultured cells. Cells treated with liposome entrapped, labelled DNA were washed with cold PBS thoroughly and suspended in SSTKNa buffer, containing 0.34 M sucrose, 0.15 mM spermine, 4 HCl, 0.5 mM spermidine. 3 HCl, 15 mM tris. HCl (pH 8.0), 60 mM KCl, 15 mM NaCl.

Radioactivity taken up by cells was determined by washing the cells on GF/C filters repeatedly with SSTKNa buffer, drying the filters and counting the radioactivity in toluene based scintillation cocktails. Isotope content of nuclei was measured similarly, except cells were lysed and washed with SSTKNa buffer containing 1% Triton X-100. For the determination of LUV encapsulated DNA, liposomes were digested with DNase I, washed and lysed with Triton X-100. DNA liberated from the vesicles was precipitated with cold 5% TCA, filtered and its radioactivity was determined as above.

Autoradiography

2×10^4 H56 cells grown on glass slides, rinsed in PBS were treated with liposomes containing radiolabelled lambda DNA for various times. The cells were washed with PBS, fixed in 3 : 1 (v/v) methanol : acetic acid followed by 5% cold TCA and repeated washings in distilled water.

H56 tk^- cells treated with Drosophila DNA encapsulated in liposomes were exposed to ^3H thymidine (10 MBq/ml, spec. act. 400 GBq/mM) one day after the liposome treatment for 24 hours. The cells were washed and fixed as above.

Autoradiography was carried out by dipping the slides into L4 Ilford autoradiographic emulsion (diluted 1 : 1 in dist. water). After 1–20 days of exposure the slides were developed stained and photographed.

DNA preparation from liposome-treated cells

CHO-Kl, H56 tk^- and SL tk^- cells were treated with liposome-entrapped HSV-TK/pBR322 or with a mixture of empty liposome and non-encapsulated HSV-TK/pBR322. For two days cells were maintained in non-selective medium which was then replaced by selective, HAT medium (Maitland and McDougall,

1977). After 1, 2, 4 or 6 days of incubation the cells were harvested and high molecular weight DNA was prepared as described by Pellicer et al. (1978). Restriction endonuclease cleavage of DNA with Bgl II was performed in the presence of 0.01% sodium azide at 37 °C overnight. The enzyme-to-DNA ratio was 1.5 units/μg of DNA and usually 20 μg of DNA was digested.

Filter hybridization

Cell DNA was tested for the presence of HSV-TK/pBR322 either by spot or Southern blot hydridization. Spot hybridization of non-digested DNA was carried out exactly as described by Brandsma and Miller (1980). ^{32}P-labelled HSV-TK probe was prepared by nick translation (Rigby et al., 1977) with a specific activity of $1–2 \times 10^8$ cpm/μg. For blot hydridization 20 μg of Bgl II digested DNAs were fractionated by electrophoresis on 0.8% horizontal agarose gel and transferred to nitrocellulose filter essentially as described by Ketner and Kelly (1976). Annealing with labelled probe was carried out according to Wahl et al. (1979).

Results

Uptake and intracellular distribution of liposome-entrapped DNA

Uptake and transport of liposome-entrapped DNA molecules by cells were studied by the use of [^{32}P]-labelled *Escherichia coli* DNA encapsulated in LUV. Radioactivity was determined in the whole cells or in the nuclei 60 min after the addition of liposomes to cells. Table 1 shows that radioactivity measured in the cells or nuclei was practically the same, independently of the doses of liposomes. [^{32}P]-labelled DNA found in the nuclei represented about 93% of total activity taken up by cells which suggests rapid transport of DNA from cytoplasm into the nucleus. (Similar results were found using chicken leukemia cells and labelled DNA entrapped in liposomes Duda and Travniček [unpublished].

Table 1

Uptake of liposome-entrapped, ^{32}P-labelled DNA by cells

Liposome + (μl)	Radioactivity (cpm) in		
	cells	nuclei	
100	10.35×10^3	9.87×10^3	(95.4%)*
50	7.90×10^3	7.31×10^3	(92.5%)*
20	5.31×10^3	4.96×10^3	(93.5%)*
10	2.72×10^3	2.38×10^3	(87.5%)*
5	1.44×10^3	1.39×10^3	(96.5%)*

+ in 1 ml of liposome suspension 2.135×10^6 cpm of ^{32}P-labelled DNA was encapsulated. Liposomes were added to H 56 cells and incubated for 60 min at 37 °C.

* Radioactivity of nuclei as compared to radioactivity of whole cells in percentage.

Intracellular distribution of radiolabelled DNA during liposome treatment was followed by autoradiography (Fig. 1). Cultured cells were treated for various times with ^{125}I-labelled, λDNA encapsulated in LUV. 0.5 min after the addition of liposomes to cells, the plasma membrane was surrounded with LUV-entrapped, labelled DNA (Fig. 1a) 10 min later DNA was found within the cells showing an almost homogenous intracellular distribution (Fig. 1b), while an additional 10 min of incubation resulted in the accumulation of labelled DNA in the nuclear or perinuclear region of the cells (Fig. 1c). These results demonstrate that liposome-entrapped DNA molecules were taken up by cells, emptied into the cytoplasma and a significant fraction of DNA was transported into the nucleus within 20 min.

The persistance and stability of the microinjected DNA

To test the presence and stability of foreign DNA, cloned DNA molecules were introduced into cultured cells by liposomes. The presence of foreign DNA was detected by nucleic acid spot hybridization after a longer period of incubation. tk$^-$ mouse and rat cells were treated with liposome-entrapped HSV-TK gene inserted in pBR322, incubated for two days in non-selective and then in selective (HAT) medium. High molecular weight DNA was isolated from cells at various times of cultivation and 1 μg from each was assayed for the presence of foreign DNA by nick translated [^{32}P]-HSV-TK/pBR322 probe. Spot hybridization of the samples (Fig. 2) showed the presence of foreign DNA in all cells treated with liposome-entrapped DNA but no hybridization was detected with DNA isolated from control cells.

The physical state of the microinjected DNA, i.e. whether it was integrated into the host genome or remained in a free form, was studied by Southern blot hybridization. DNA extracted from liposome-treated tk$^-$ rat cells was digested with Bgl II restriction endonuclease cleaving the circular HSV-TK/pBR322 molecule only at a single site. DNA fragments, separated by gel electrophoresis and transferred to nitrocellulose filters were hybridized to highly labelled ^{32}P-HSV-TK/pBR322 probe (Fig. 3). Fragments equivalent to or larger than the liposome-injected DNA were present in the cells one or two days after the liposome treatment, corresponding to the free and probably to the integrated forms of plasmid DNA. A few days later the pattern of fragments has changed suggesting the disappearance of the free form, leaving only integrated sequences in the nuclei.

Expression of genes of liposome-injected total genomic DNA in mammalian cells

Gene expression of total genomic DNA sequences extracted from Drosophila melanogaster, chicken red blood cells and human embryonic liver was investigated.

Expression of Drosophila DNA entrapped in liposomes was tested in tk$^-$ mouse cells by autoradiography. Cells were treated with 'empty' and DNA-loaded liposomes and then incubated in HAT medium containing [^{3}H]-thymidine. After one day of cultivation (allowing time for phenotypic expression), the autoradiography of control and DNA-treated cells showed a striking difference in [^{3}H]-thymidine incorporation (Fig. 4). In cells treated with empty liposomes no radio-

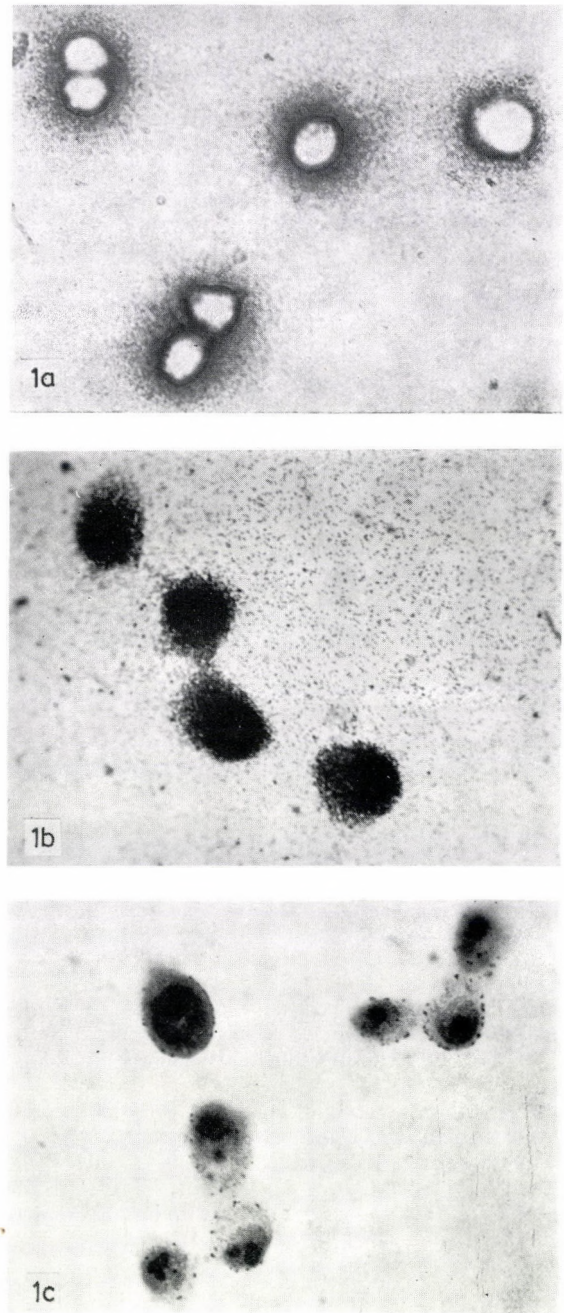

Fig. 1. Cell-liposoma interactions and intracellular distribution of ^{125}I labelled DNA followed by autoradiography CHO-K1 hamster cells were treated with liposomes entrapping ^{125}I labelled lambda phage DNA. Incubation times were: a, 0.5 min; b, 10 min; c, 20 min

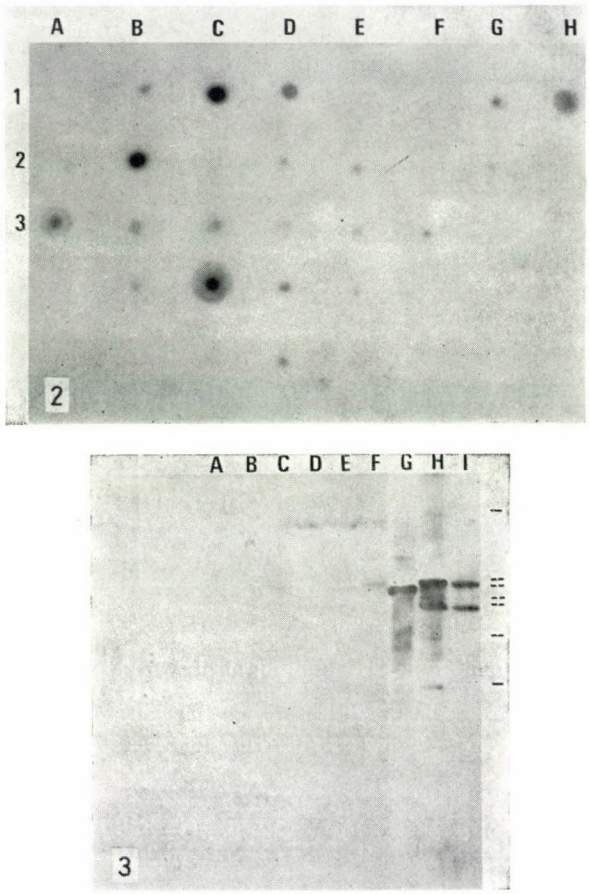

Fig. 2. Spot hybridization of DNAs derived from liposome-treated cells. ^{32}P-labelled HSV-TK/pBR322 was used as a probe. 2A—2G, DNA from SLTK cells 1, 2, 3, 4, 5, 6, 8 and 9 days after treatment with liposomes containing HSV-TK/pBR322; 3B—3E, DNA from H56 cells 2, 4, 6, and 8 days after treatment with liposomes containing HSV-TK/pBR322; 1A, untreated SLTK; 1C, untreated H56; 2H, SLTK and 3A, H56 treated with a mixture of empty liposomes and HSV-TK/pBR322; 1B, HSV-TK/pBR322

Fig. 3. Presence of HSV-TK/pBR322 sequences in cells treated with liposome-entrapped HSV-TK/pBR322. The hybridization profiles of 20 μg of Bgl II-cleaved DNA from tk$^-$ mouse cells (lane A) and cells treated with a mixture of empty liposomes and HSV-TK/pBR322 (lane B) are shown along with the Bgl II patterns of DNAs derived from cells treated with liposome-entrapped HSV-TK/pBR322 for 1 day (lane C), 2 days (lane E), 4 days (lane F) and 6 days (lane D). HSV-TK/pBR322 and markers are shown in lane G and lane H-I, respectively

activity was found while a high amount of [^{3}H]-thymidine was incorporated into the DNA of cells microinjected with Drosophila DNA (Fig. 4b). Incorporation of [^{3}H]-thymidine reflects thymidine kinase enzyme activity, probably due to the

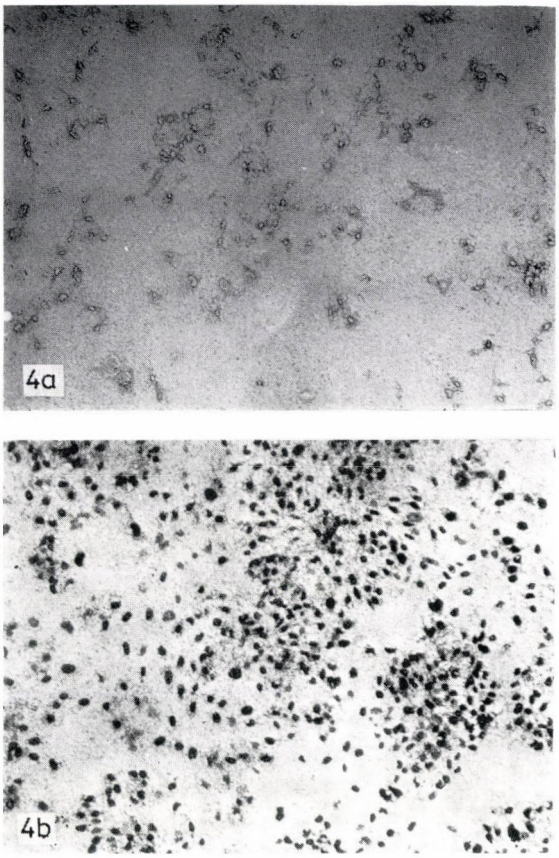

Fig. 4. Thymidine kinase gene expression in treated cells tk^- mouse cells were treated with Drosophila DNA containing liposomes and one day later thymidine kinase activity was detected on the basis of ^{3}H-thymidine incorporation. a, cells treated with "empty" liposomes; b, cells treated with liposomes containing Drosophila DNA

transient expression of Drosophila tk gene. No stable tk^+ transformants were produced after a 3 weeks period of incubation in HAT medium.

Transient expression of Drosophila DNA was demonstrated also in CHO-K1 pro^- cells by prolonged survival of cells in proline free medium (Fig. 5). Cells treated with empty liposomes showed typical survival curves while cells microinjected with DNA showed a somewhat increased life-time.

While the temporary expression of microinjected genes could be detected in a large fraction of treated cells, stable genetic transformation of recipient cells occurred only at a rather low frequency. Transformant colonies were produced only if cells were microinjected with avian or mammalian genomic DNA. 200 μg high molecular weight DNA purified from chicken red blood cells or human em-

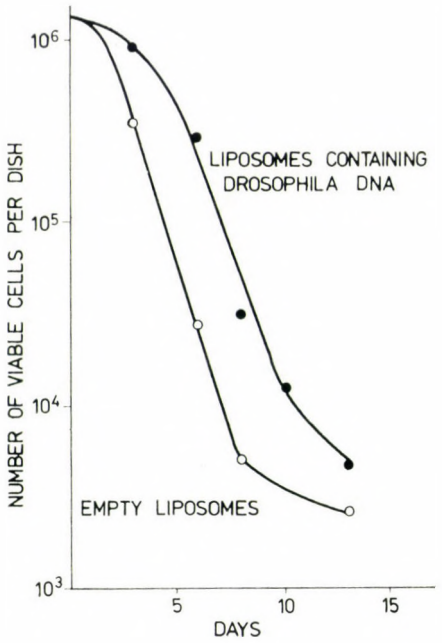

Fig. 5. Survival of CHO-K1 pro$^-$ cells in proline deficient medium after liposome mediated DNA transfer. Cells were treated with "empty" and Drosophila DNA containing liposomes (for one hour) and kept on medium lacking proline. Transient gene expression in DNA treated cells increased survival time with 2—3 days

bryonic liver were encapsulated in liposomes prepared from 1.9 μmol phosphatidyl serine per ml and 100 μl of liposome suspension were added to each petri dish containing 5×10^6 CHO-K1 pro$^-$, or mouse or rat tk$^-$ cells. After cultivation of the cells in proline free or HAT medium, respectively, pro$^+$ and tk$^+$ colonies were observed at a frequency of approximately 10^{-5} (above rate of 10^{-7} to 4×10^{-7} found in case of untreated, control cells).

Discussion

The potentional usefulness of liposome-entrapped genetic material for transformation of cultured cells highly depends upon the uptake of encapsulated material and on the fate of microinjected nucleic acids within the cells. We have demonstrated that liposome-entrapped DNA molecules from different sources were effectively taken up by cells and transported from the cytoplasm of cells into the nucleus. 20 min after the addition of liposomes to cells a high proportion of foreign DNA accumulated in the nuclear and perinuclear parts of the cells, and 40 min later approximately 93% of the labelled foreign DNA was localized within the

nucleus. The use of cloned DNA molecules demonstrated the survival of microinjected DNA molecules for at least for 8 days. The foreign DNA sequences seemed to be integrated, having molecular weights higher than the introduced plasmid DNA. (The presence and state of foreign DNA were investigated only in a relative short period of incubation since 8 days after the liposome treatment almost all cells died, providing insufficient amounts of cells for extraction of host DNA.)

Temporary expression of foreign genomic DNA was observed in a great proportion of liposome-treated cells; however, stable genetic transformation of mammalian cells produced by avian or mammalian DNA was a relatively rare event. The fact that no transformant colonies were observed after treatment with Drosophila DNA is in agreement with the results of DNA-mediated transformaton of mammalian cells, although our data indicated a transient expression of Drosophila genes.

On the basis of our results we assume that nucleic acids entrapped in liposomes can be applied for transformation of cultured cells and liposome-mediated microinjection of DNA provides an alternative, probably generally applicable technique for introduction of specific genes into cultured cells.

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Wandering of a Black Sheep

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Once upon an early period in my scientific carrier Straub had taken side with me in a sharp, heated discussion in the old Institute of Medicinal Chemistry. It caused some surprise among the older members of that famous Institution. To annoy them more in the late afternoon of the same day Ágnes Ullmann devised a paper bib for me with the inscription "Daddy's favourite". Her intention was not quite clear but it made a long-lasting impression on me. I definitely wanted to inherit some of the bad traits of our "Daddy's" character. First of all his persistence: by introducing an unexpected idea finally he always managed to turn a completely fruitless work into an apparently most thoroughly devised triumph envied by many. Also his playful passion with which he approached scientific problems whether the conditions to solve them were given or not. And strangely enough, after some time the given conditions turned out to be the most appropriate in supporting his ideas. I needed long years of practice before manifesting my inheritance in a modest way.

I began working on the regulation the *B. cereus* penicillinase. At the time, the efforts of most scientists in the field were concentrated on studying the synthesis of β-galactosidase in the well-known *E. coli*, the unique bacterium on which the whole empire of the new biology was founded. Attention was directed mostly to the connection of ribonucleic acids and protein synthesis. Later it was discovered that β-galactosidase and the penicillinase systems differ strongly in this respect, because β-galactosidase synthesis is controlled by high turnover mRNA while the penicillinase system is characterized by a stable mRNA. Therefore, the latter seemed to be an appropriate first model for studying eukaryotes in which protein synthesis is also directed by stable mRNAs.

We have found a new type of enzyme induction caused by concentration electrolytes. *B. cereus* growing on an electrolyte containing medium secretes large amounts of penicillinase in the absence of a specific inducer (penicillin). The aspecific character of this induction was a great challenge for both theory and methodology. We proved that the electrolyte-caused induction was due to the conformational change of the natural inducer binding protein (Csányi et al., 1967a), which was assumed to be the main regulatory factor of penicillinase synthesis. We could remove the penicillin-binding protein from the bacterial cell wall, and confirmed both the regulatory function of this particular molecule and its role in stabilising the penicillinase m-RNA (Csányi et al., 1971a). These studies culminated in the

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construction of a new regulatory model of penicillinase synthesis. The "firmator" model was the first and only model which interpreted all the available experimental data on penicillinase and firmly supported the central role of stable m-RNA in the regulatory mechanism. [For some early reactions see Collins (1971).]

Besides the studies on the regulatory mechanism of penicillinase synthesis we also studied connections between the chemical properties, e.g. conformation, the position and oxidative state of the amino acid side chains and the enzymatic activity of the penicillinase protein. We established the chemical basis of the peculiar iodine sensitity of penicillinase enzyme (Csányi et al., 1970a, 1971b, 1971c). We also studied the correlations of the enzyme structure and the effects of synthetic penicillin derivates (Csányi et al., 1970b) on which the pharmacological activity of the latter is based.

At this time I believed my career in biochemistry led straight up to the skies, but when the "Golden Cage" of the Biological Center of the Academy was set up for my peers at Szeged, suddenly I said good-bye to biochemistry. This was appropriately dramatised by my friend Peter Friedrich who, reviewing Hungarian Biochemistry, wrote: "Stop press news: Csányi and his co-workers have packed up the penicillinase work and embarked on an entirely different subject, the biochemistry of learning and the biochemical aspects of behaviour genetics. Does fortune favour the brave?" (Friedrich, 1973). A considerable time has passed since, so now I can answer your question Peter: If *anybody* favours him at all, it is certainly Fortune.

While I was working with bacteria I always had some misterious idea that I would feel much more confortable in the company of animals. The first step in this direction was made earlier when I had a Ford Fellowship at Harvard for studying enzyme induction in mammals with Olga Greengard. We have shown unquestionably that upon induction of tyrosine aminotransferase by various hormones a stable mRNA is synthesised (Csányi et al., 1967b, Csányi and Greengard, 1968). A further step towards the newly chosen field also involved biochemistry. Invited to the New York Center for Neurochemistry I worked with Abel Lajtha on the axoplasmic transport of free amino acids in the nervous system. Free amino acid transport was questionable at this time because of the contradictory data. Some experiments upported the free amino acid transport by axoplasmic flow, but many researchers argued against the possibility of this kind of transport on the grounds that the secondary breakdown of transported proteins were not ruled out by the experiments. However, using nonmetabolising amino acids we have shown that free amino acids really are transported and the possibility of their secondary origin has been excluded (Csányi et al., 1973a).

The rise of ethology made its impact on biology during the golden years of the seventies and I realised that I wanted to be in ethology, especially in behaviour genetics. First without a clear perspective, just to gain experience I began to deal with imprinting.

Young individuals of several precocial species stay near their parents for a certain period after hatching; they follow the parents if necessary, and are able

to distinguish them from other adult individuals of the species. The part of this complex of activities through which the young animals acquire, by a very brief exposure, a tendency to follow or approach the parents or an appropriate stimulus source on a test made at a later time, was termed by Lorenz as a special kind of learning: "imprinting". In our experiments with chickens we found that the approach response to a relatively weak imprinting stimulus can arise by two different mechanisms. Some chickens approached the object of the stimulus within a few minutes, others needed a considerably longer stimulation, essentially a conditioning to elicit the approach response. The two mechanisms which we termed "prompt approach" and "conditioned approach" are distinguished on the basis of the different conditions required for eliciting them (Csányi et al., 1973b).

Biochemical approach flashed up again in some experiments. The effects of puromycin and cycloheximide on memory of birds were examined in an imprinting process. Prompt approach response of chicks to an imprinting stimulus was inhibited neither by puromycin nor by cycloheximide, two potent inhibitors of protein synthesis. But conditioned approach response was almost completely prevented by intracerebrally administered puromycin. Puromycin also influenced the chicks' stimulus recognition in a discrimination test, without impairing their ability and readiness to make a choice between two stimuli (Gervai and Csányi, 1973). The same year in which these results on imprinting were published I was invited to L. Eötvös University's Faculty of Natural Sciences to organise the Laboratory of Behaviour Genetics. I decided to work with fish, primarily with the paradise fish *(Macropodus opercularis)* which has a short generation time, large number of offspring and is convenient to work with in the laboratory. My long-term project reflected a new approach in behavioural genetics analysis in that we try to analyse the genetic architecture of natural components of behavior.

It was clear that we had to work in two loosely connected fields. First we needed genetically homogeneous strains of paradise fish for the behavioral study and we had to work out the proper ethological methods of observing fish behavior which would be suitable for genetical studies.

But first of all we badly needed money because our budget at the University was scarcely enough for fish food. This was the first time for fortune to favour the brave. I met with István Tölg who has just set up a new warm-water fishery designed and directed by himself. When I spoke to him about our plan to obtain genetically homogeneous *Macropodus* in the shortest possible time by applying gynogenesis, a peculiar kind of parthenogenesis, he immediately realised its applicability in commercial fish breeding and generously offered long time support and research facilities. His condition for the support was that we should work with carp. I accepted the offer and so we made a detour in research.

The phenomenon of artificial gynogenesis has long been known, but the extremely low yield of gynogenetic offspring has made it a laboratory curiosity. Theoretically, parthenogenetic propagation promised to be a very effective way of getting genetically homogeneous lines very quickly. After some hard years we succeeded in mass production of gynogenetic carp (Nagy et al., 1978).

To make the intercrossings of gynogenetic carp lines possible, we worked out the method of phenotypal sex-transformation of genetically female carps for producing fertile males (Nagy et al., 1981). The inbreeding of carp by gynogenesis and intercrossing the homogeneous lines resulted in a new commercial breeding program in Hungary. The Fisheries Research Institute is introducing our hybrids right now into commercial production. The new hybrids are producing 20–40% more meat than the presently used best lines (Nagy et al., 1984). We developed a short-term laboratory system for the evaluation of carp growth in ponds (Nagy et al., 1980) and we also produced high yield triploid carps, which hold promise for commercial breeding, too (Gervai and Csányi, 1978, Gervai et al., 1980a).

Beside the applied research of carp genetics (Nagy and Csányi, 1978; Gervai and Csányi, 1978) we used carp in studies of classical genetic analysis (Nagy et al., 1979; Gervai et al., 1980a) and immunogenetics (Nagy et al., 1983). Some of our studies provided some contribution to the understanding of the mechanism of genetic homogenisation by gynogenesis. We showed that while several subsequent gynogenetic propagation results in isogenic populations suitable for breeding, contrary to the general expectation the inbreeding coefficient remains below a certain value. It is because genes of high recombination frequency tend to remain heterozygous (Nagy and Csányi, 1982). To overcome this effect and to produce truly inbred strains, we worked out a new breeding system. Based upon the distribution of recombination frequencies, a strategy of alternating gynogenesis and sibmating with sex-transformed gynogenetic males results in selfing type breeding by which high level of homozygosity can be achieved (Nagy and Csányi, 1984). These results are appropriately valued by international reviewers (Cherfas, 1981; Kirpichnikov, 1981; Thorgaard, 1983).

Different theoretical aspects and practical use of inbreeding and gynogenesis were dealt with in a comprenhensive review (Csányi, 1983).

Working on carp gynogenesis took much time and energy away from our basic research but we have gained some knowledge which was used in producing gynogenetic lines from *Macropodus* (Gervai and Csányi, 1984). For an appropriate genetic control over the newly developed *Macropodus* strains we identified several biochemical markers in this fish (Monostory et al., 1983). Our aim with the gynogenetic lines besides getting inbred lines, was to develop *recombinant inbred* (RI) strains from *Macropodus*. RI strains were first established in mice and became most sophisticated tools in vertebrate genetics. These strains allow one to arrive at genetic linkage and models for genetic determination of particular traits in comparatively short periods of time. RI strains are derived from the cross of two unrelated but highly inbred progenitor strains. This cross is then maintained independently from the F_2 generation with strict inbreeding. The procedure fixes the change recombination of genes in generations following the F_2. The resulting battery of strains can be looked upon in one sense, as a replicable recombinant population. In actual experimental use, an indication of the approximate number of effective loci determining differences in a trait, is obtained from the number and range of distinctive phenotypic groupings in relation to the progenitor strains.

The location of the groupings in relation to the progenitor strains and their F_1 also has the potential of revealing dominance and epistatic gene action. With comparisons of the strain distribution patterns of different traits it is possible to obtain cues of potential linkage relationship. Nevertheless there are quite a few problems in applying this new technique. The most annoying problem is the length of time needed to establish an RI strainbattery. To breed the forty generations required to reach the appropriate level of homozygosity takes many years, even in the mouse. It seemed quite appealing to apply gynogenesis for shortening the necessary time. According to our calculations one gynogenetic generation is approximately equal with 6–8 generations of sibmating in term of genetic homogeneity. We just finished the development behavioural testing of a Macropodus strain battery of 15 strains (Gervai and Csányi, 1985). But before we are going in to details of that study some words are necessary about our behavioural experiments.

First there were series of pilot experiments in which we tried different methods (Székely and Csányi, 1978; Kabai and Csányi, 1978a; Vadász et al., 1978a; Székely et al., 1978).

We defined two classes of observational parameters. The first class is the so-called behavioural-test, widely used in experimental psychology. The animal to be tested is put into a highly artificial and carefully controlled environment and certain predetermined parameters of its behaviour are measured. We refer to this test by various denominations such as "open field behavior", "curiosity", etc.

The second class of parameters are the "action units". An action unit is a set of behavior patterns defined by ethological criteria. For example the *creeping unit* is a characteristic slow movement. The fish, closely clinging to the bottom, is propelled forward only by pectorial fin fanning. Dorsal, pelvic, anal and caudal fins are closed. Creeping may be observed under a wide range of environmental circumstances including natural ones (Csányi et al., 1985a, 1985b). In order to analyse action units the fish are placed in various, more or less natural environment, observed through closed TV circuit and the actions are recorded with the help of a computer. We found that action units are genetically controlled and as behavior parameters are appropriate for genetic analysis (Kabai and Csányi, 1978b; Vadász et al., 1978b).

We made a large comparative testing program on the battery of the gynogenetic recombinant inbred (GRI) strains of the paradise fish. Ninteen independent parameters measured in the various behaviour tests and about the same number of action units were recorded in hundreds of the animals of the GRI groups. The statistical evaluation is in progress and preliminary results show that most of the test parameters as *traits* are polygenic and only a few of them are worth for further analysis. Among the action units there are quite a few in which the variability between the recombinant strains appears to be controlled by one or a few genes (Gervai and Csányi, 1985, 1986, Csányi and Gervai 1986a, 1986b).).

We hope that with the help of this genetic analysis a clear identification of the natural units of behavior can be achieved and a way to etho-genetical analysis will be opened up.

In recent experiments we studied fish behavior in a more complex situation involving also learning behaviour. In seminatural environment and an aquatic Skinner-box we observed the behaviour of the paradise fish in the presence of natural predators a pike or members of peaceful species and various fishlike dummies. In some experiments mild electric shock was applied as a source of pain.

The results of the various observations and experiments are as follows: When the paradise fish meets a member of another species of fish, an innate exploration is induced which is waning quickly upon repeated meetings. However, if the approached fish or fish-like object reacts and injures of frightens the paradise fish, then avoidance response is elicited and fixed by a quick learning process. The avoidance response can be elicited again whenever the members of the same species appear.

The results in passive avoidance learning paradigm indicated that appropriate stimuli, i.e. fish of another species and fish-like dummies enhance the effect of mild electric shock very effectively. We found that specific key-stimuli are necessary for a proper avoidance conditioning process.

To account for these observations we constructed a new hypothesis, which was based on the assumption that the brain was *modelling* its environment. The central feature of this hypothesis is the genetically determined species-specific *key stimulus*. Appearance of such stimuli in the environment of the paradise fish results in immediate orientation and turning on an *identification process* manifested in approach and thorough exploration. If the carrier of the key stimuli is passive then the exploratory behaviour slowly diminish by way of simple *habituation*. If the carrier of the key stimuli interacts with the paradise fish and causes fear or pain then *species-specific defensive reaction* becomes activated and the *exact features* of the carrier are fixed in *memory* by S-S type association. In the case of recurring of the same stimulus carrier the defense reaction becomes elicited by its *representation* in the memory *without* previous exploration. We call this hypothesis *interactive learning*, because learning is the result of the paradise fish's active search for the appropriate object.

In terms of the modelling function of the brain, the brain of the paradise fish is assumed to contain some kind of "wired in" representation of the key stimuli, which makes the identification possible. It also has an innate tendency to explore, directed by these innate representations and a proper mechanism to elicit species-specific defense reaction, if fear and pain come upon it. The function of model building is to provide this dynamic mechanism with precise data about the carriers of key stimuli. In nature where peaceful and predatory carriers of key stimuli can be found as well, certain amount of learning results in the representation of the most important features of the given environment in the brain of the paradise fish in the form of a model. The representation of peaceful species manifest themselves in *restraints*, the representations of predators in *active avoidance behavior* (Csányi, 1985a, 1985b, 1986).

Our ethologically oriented research also produced some practical results. By observing action units, the state of the nervous system of the animal can be

described very exactly (Nagy et al., 1986). In cooperation with an Italian psychopharmacological group we use ethological methods to evaluate the effects of various opiates on the nervous system (Csányi et al., 1984; Dóka et al., 1985).

If one is working on widely different projects as ranging from genetics to ethology, it is a compelling necessity to build up a theoretical framework which contains every important aspet of such projects. Nowadays genetics is based on an evolutionary approach and model-building function of the animal brain is also understandable only in terms of behavioral evolution. For a long time my main theoretical interest has laid in evolution. I would like to comprehend all main aspects of life and to bring into a unified conceptual framework the molecular, cellular and organismic levels of evolution not only with behavioral and ethological, but also with the cultural and technical levels, which are not generally incorporated into the classical concept of the Darwinian theory. The first sketch of a general theory of evolution was published in Hungarian (Csányi, 1978). A concise English paper followed it (Csányi, 1980). This paper was reprinted upon the request of the Institute of General System Research in their yearbook (Csányi, 1982a). The English translation of the book was published, too (Csányi, 1982b). The first review seems reasonable (Vine, 1983).

My latest publications in this field describe *autogenesis*, a newly developed conception based upon the earlier general theory (Csányi 1985c, Csányi and Kampis 1985, Kampis and Csányi 1986). Autogenesis is a theoretical model which has been introduced for the description of the spontaneous emergence of replicative organization in compartment systems like living and social systems. It was suggested that imperfect replication – a copying process achieved by a special network of functionally interrelated components and component-producing processes that produces the same network as that which produced them – characterizes such systems. The information used in this copying process, whether it is stored by special means or distributed in the whole system is called replicative information. During autogenesis, replicative information increases in the system and compartments form. A compartment is the co-replicating totality of components. The end state of autogenesis is an invariantly self-replicating organization which is unable to undergo further intrinsic organizational changes. Levels of evolution emerge as a consequence of the relative autonomy of the autogenetic unities. On the next level of organization they can be considered as components with functions and a new autogenetic process can commence. Thus evolution proceeds towards its end state through the parallel autogenesis of the various levels.

In terms of application, ontogenesis of multicellular organisms is dealt with in detail as an autogenetic process as is the autogenesis of the biosphere (Csányi and Kampis 1985) and the global bio-social system (Csányi and Kampis 1986).

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Translational Coupling at the Intercistronic Boundary of an Artificially Constructed Operon in Escherichia coli

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By using recombinant DNA techniques an artificial operon was constructed that codes for two fusion proteins under the control of the beta-lactamase promoter of plasmid pBR322. The two proteins are: 1. beta-lactamase-trp B (43 kd) 2. trpA-beta-galactosidase (120 kd). Frameshift mutations in the N-terminal region of the first gene resulted in a dramatic reduction in the synthesis of the protein coded by the second gene. This strong polar effect could not be accounted for by correspondingly lower level of the distal region of the messenger RNA, only by "translational coupling" due to the overlap of the termination codon of the first gene with the initiation codon of the second gene. It was concluded that the strong "translational coupling" observed in this artificial operon can be generally used to ensure coordinated high-level synthesis of proteins in operons constructed by recombinant DNA techniques.

Operon polarity is a well known phenomenon in prokaryotic molecular biology. It means that nonsense mutations in promoter proximal genes of various operons lead to decreased expression of the distal genes of the same operons, although these genes do not contain any lesion. Ample evidence suggests that the primary cause of this effect is the dissociation of translating ribosomes from the polycistronic message at the mutant site. As a consequence, the "naked" messenger is exposed to either more rapid decomposition starting from the 3'end, or premature termination at secondary, rho-dependent termination sites (Adhya and Gottesmann, 1978). In other words, the polarity is essentially transcriptional; decreased expression of distal genes is due either to decreased synthesis, or faster decomposition of this part of the polycistronic messenger. In some exceptional cases however the existence of a different mechanism, called "translational coupling" has been demonstrated (Schümperli et al., 1982; Oppenheim and Yanofsky, 1980). It has been shown that if the translational stop and start signals of two adjacent genes of an operon follow each other very closely, or even overlap, nonsense mutations in the proximal gene result in strong polar effects in the neighbouring gene, without strongly affecting the other, more distal genes of the operon. In these cases the transcriptional explanation has been ruled out and it was proposed that the underlying mechanism is the tight coupling of the translation of the two adjacent genes. It has also been suggested that this translational coupling is an important regulatory mechanism which ensures the equimolar production of the proteins coded by the adjacent two genes. In their paper reporting translational coupling between the trp E and trp D genes, Oppenheim any Yanofsky write: " . . . it is

conceivable therefore that translational coupling between trp B and trp A, similar to that we observe between trp E and trp D could be demonstrated".

In this paper we demonstrate the existence of just this coupling. Moreover by demonstrating the efficient coupling at the trp B- trp A boundary in a different context, in an artificially constructed operon, we also show that this (or similar) boundary region(s) might serve as portable coupling elements in operons constructed by recombinant DNA technology to ensure an equimolar synthesis of various proteins coded by the operon.

Materials and methods

Bacterial strains and plasmids

Escherichia coli HB101 (Boyer and Rouland-Dussoix, 1969) the lac$^-$ indicator strain ED8800 (Murray et al., 1977) and the minicell forming DS410 (Dougan and Sherratt, 1977) were used. For plasmid construction plasmids pBR322 (Bolivar et al., 1977) and plac23 (Boros et al., 1983) served as starting materials.

Enzymes: All restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were prepared in this laboratory by established protocols. S_1 nuclease was from Sigma, Bacterial alkaline phosphatase from Worthington, DNA polymerase Klenow-fragment from Boehringer.

Recombinant DNA methods

Plasmid DNA was isolated from *Escherichia coli* HB101 after chloramphenicol amplification by the method of Clewell (1972). The crude plasmid was further purified by gel filtration chromatography on Sephacryl S-1000. Transformation was carried out according to Lederberg and Cohen (1974). Ligation of DNA with cohesive ends was performed as described by Covarrubias and Bolivar (1982). Attachment of synthetic linkers was done according to the manual of Maniatis et al. (1982). All restriction endonuclease digestions were performed as recommended by New England Biolabs. For the removal of single-stranded ends DNA (at 20 μg/ml) was incubated with S_1 nuclease (5 units/μg DNA) in 30 mM Na-acetate, 1 mM $ZnSO_4$, 0.3 M NaCl, 5% glycerol at pH 4.6 for 15 min at room temperature. DNA fragments were isolated from agarose gels by electroelution.

Beta-galactosidase assay

Beta-galactosidase positive transformants in the lac$^-$ host ED8800 were screened using McConkey agar indicator plates. For quantitative assay the method described in Miller's manual (1972) was used.

Translation in minicells

Minicells were prepared according to Meagher et al. (1977) from *E. coli* DS410, transformed with the appropriate plasmids. Labelling was with ^{35}S-L-Methionine (Amersham, 800 Ci/mmole) for 30 min (10 μCi/sample).

Labelled proteins were separated on 8% acrylamide-SDS gels by the method of Laemmli (1976).

DNA-sequencing

The Maxam-Gilbert technique was used (1980). Labelling was done by repair synthesis at the 3' termini, using DNA-polymerase Klenow fragment in 50 mM K-phosphate (pH 7.4), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 μM dCTP, dGTP, TTP and 0.5 μM alpha-^{32}P-labelled dATP. (It was prepared by the method of Walseth and Johnson, 1979.) DNA fragments were isolated from acrylamide gels by electroelution.

Hybridization

Isolated DNA fragments were dissolved in water at 500 μg/ml, denatured with NaOH, neutralized, adsorbed and fixed to nitrocellulose as described in the manual of Maniatis et al. (1982). For the preparation of labelled RNA HB101 cells transformed with the appropriate plasmids were grown in glucose-minimal medium to an OD_{600} 0.6, then 20 μCi/ml ^{3}H-uridine (Chemapol, Prague) was added. After 90 s cells were poured onto crushed ice, centrifuged, and resuspended in 50 mM Na-acetate (pH 5.2) – 0.5% SDS. After three successive phenol extractions RNA was precipitated with 2.5 vol ethanol in the presence of 0.3 M Na-acetate, centrifuged, dried and finally dissolved in 0.1 ml water. Prior to hybridization filters were boiled in water for 1 min. Hybridization was carried out in 2 × SSC for 18 hours at 65 °C with 6–8.10^5 cpm ^{3}H-RNA/filter. Filters were washed twice with 2 × SSC, incubated for 1 hour at room temperature with 20 μg/ml RNase in 2 × SSC and washed again three times with 2 × SSC before measuring the radioactivity. All assays were done in triplicate and hybridization to RNA isolated from plasmid-free HB101 served as control.

Results and discussion

Our experimental system was a recombinant plasmid, carrying an artificially constructed operon. It consisted of two fusion genes connected with the natural boundary between trp B and trp A. It has previously been shown that at this boundary the two genes overlap, the last nucleotide of the trp B stop codon is the first nucleotide of the trp A start codon (Platt and Yanofsky, 1975). The first (promoter-proximal) gene was fused from the promoter, and translational start region of the beta lactamase gene of the pBR322 plasmid, and the 3' part of the trp B gene. The two coding regions were fused in phase, with a HindIII linker in between. The linker provided a unique restriction site by which frame-shift mutations could be easily generated. The second (distal) gene was fused from the 5' part of trp A and most of the lac z (beta-galatosidase) gene of *E. coli*. As only the nonessential N-terminal amino acids of the beta galactosidase protein were replaced by the 5'-terminal (N-terminal) trp A peptide, the fusion product retained its beta galactosidase activity, thus its synthesis could be detected on indicator plates and easily measured

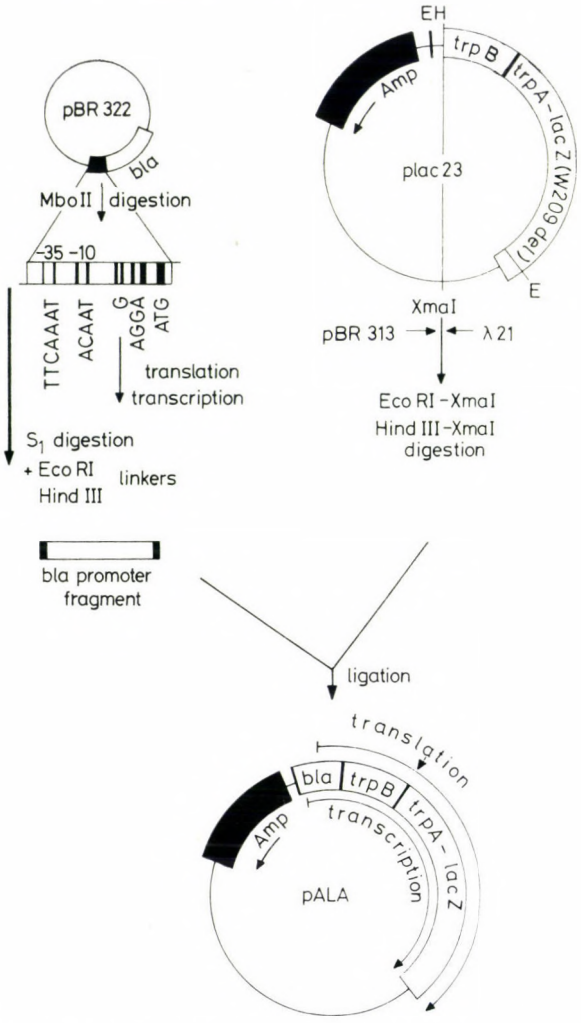

Fig. 1. The construction of pALA1. Only the relevant restriction sites are shown. (E = EcoRI, H = HindIII, X = XmaI)

quantitatively. The construction of this artificial operon is schematically illustrated on Fig. 1. Most of the sequence originated from the E. coli strain W209, a deletion mutant in which the trp and lac operons were fused. This fused operon was part of the promoter-detecting vector lambda 21 (Holowachuk et al., 1980) and used for the construction of the pBR313 derived plasmid plac23 (Boros et al., 1983). plac23 contains part of trpB, the trpB-trpA boundary, and the 5' end of trpA fused to lacZ, but this whole sequence is not expressed because of the absence of any promoter. The promoter originated from plasmid pBR322 in the form of a 196 bp

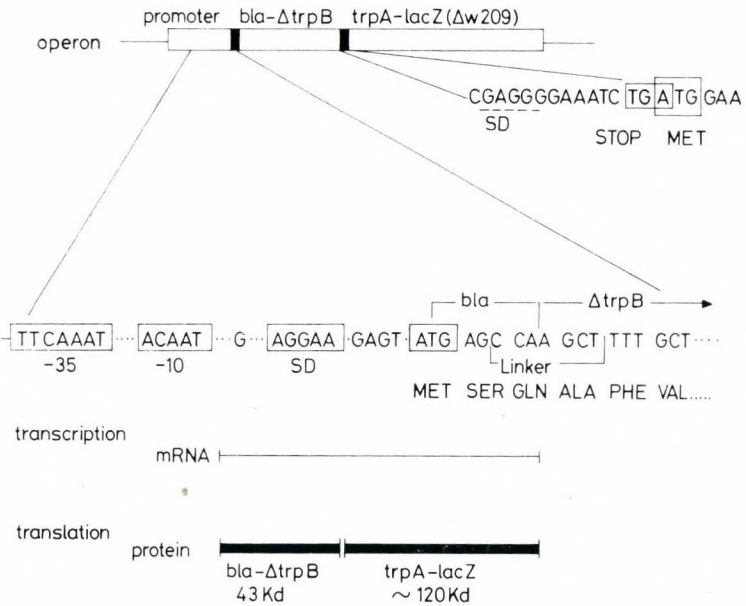

Fig. 2. Schematic illustration of the fused operon of pALA1

MboII fragment (map position 4157–4353) (Sutcliffe, 1977). This fragment was isolated, treated with S_1 nuclease and ligated with synthetic EcoRI and HindIII linkers. After digestion with EcoRI and HindIII, this fragment was ligated to HindIII-XmaI and EcoRI-XmaI digested plac 23. The ligated DNA was then used to transform the lac$^-$ E. coli strain ED8800. Amp^R lac$^+$ colonies were selected and further analysed. One of these Amp^{R}lac$^+$ clones (termed pALA-1) exhibited a very high level of beta-galactosidase (2500 $\pm$ 400 Miller units). The structure of this plasmid was analysed first by restriction analysis and Southern blotting, then with sequencing the relevant region by the Maxam–Gilbert procedure. Its structure is illustrated on Fig. 2. It was shown that in this plasmid, the promoter, and the 5'-end of the beta-lactamase gene was connected with the HindIII linker to the 3'-end of the trpB gene in the appropriate reading frame, thus the fused gene was translated, and this permitted the high-level expression of beta-galactosidase. This was confirmed by analysing the proteins synthetised in a "mini-cell" system programmed by the plasmid. Figure 3 shows that the predicted 43 kd protein corresponding to the beta-lactamase-trpB fusion protein, and the 120 kd protein corresponding to the trpA-lacZ fusion are indeed being synthetised.

Nonsense mutations in the first gene of the artificial operon of pALA-1 are easy to generate by causing a fragmeshift. For this purpose, the plasmid was linearized at the unique HindIII site, the resulting protruding ends were digested with S_1 nuclease, and the blunt-ended DNA was recircularized with T4 DNA-ligase.

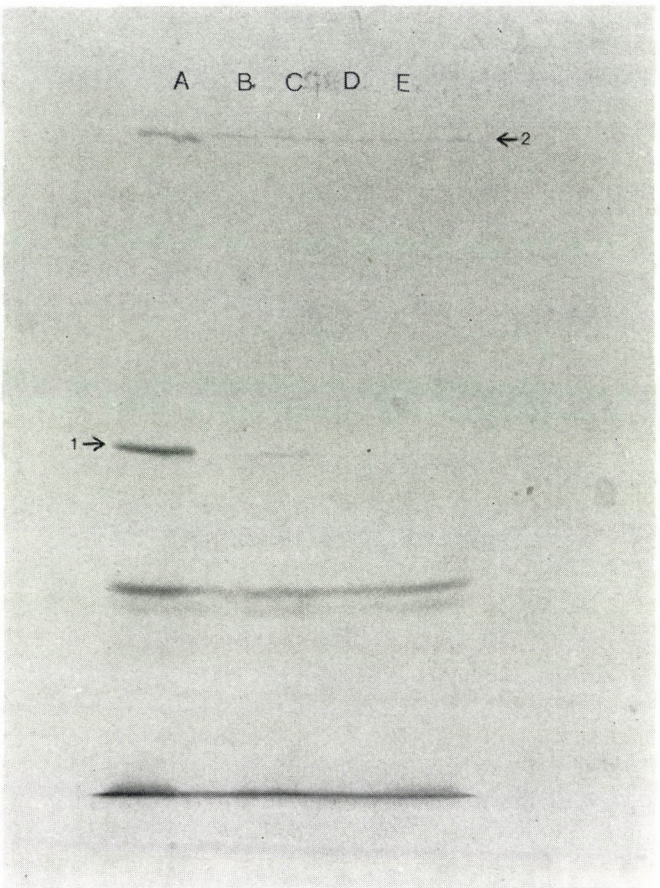

Fig. 3. Proteins synthetised in minicells programmed by recombinant plasmids. Autoradiogramm of ^{35}S-labelled proteins, separated on a 8% polyacrylamide-SDS gel. Slots A and C: plasmid pALA1. Slots B, D and E: recombinant plasmids pALA1ΔH_1 19, pALA1ΔH_1 30, pALA1ΔH_1 32 (frame-shift mutants). The arrows indicate the 43 Kd and 120 Kd proteins

This 4 bp deletion generated a frame-shift, and thus a terminator codon near the beginning of the trpB sequence (Fig. 4). As shown in the minicell system, this mutant did not produce the 43 kd protein, and the synthesis of the 120 kd protein was also reduced. The beta-galactosidase measurement in cell extracts revealed a drastic approximately 20-fold reduction to about 114 ± 18 Miller units (Table 1).

In order to distinguish between the possibility of transcriptional or translation origin for this extremely strong polar effect, we tried to determine the level or messenger RNA. For this purpose the 5 kb EcoRI fragment of plac23 encompassing the whole trp-lac region and the 1.98 kb HpaI-EcoRI fragment of the same plasmid, containing only lacZ sequences were isolated, fixed on nitrocellulose and

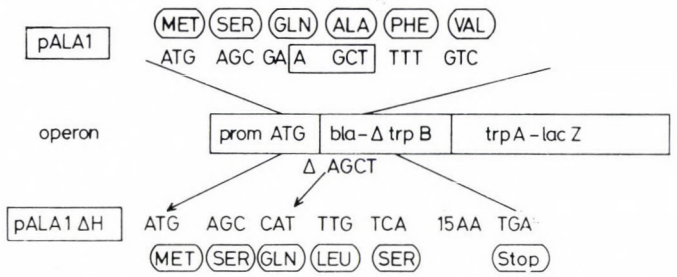

Fig. 4. Generation of the frame-shift mutation in pALA1

hybridized to ^{3}H-uridine labelled RNA isolated from HB101 E. coli cells transformed with either pALA-1 or the frameshift mutant. These hybridization experiments are summarized in Table 1. It can be seen that although the frameshift causes a slight drop in the level of messenger coded by the distal gene, this cannot possibly account for the 20-fold decrease in protein level.

Thus it must be concluded that in this case the strong polar effect is due to translational coupling. Because of the overlap of the trpB terminator and trp A initiator codons, trp A translation can be efficiently initiated only if trpB translation is properly terminated. If trp B translation is prematurely terminated (as in the frameshift mutant) ribosomes fall off from the messenger. This leads to a slight decrease in messenger level, but the far more dramatic result is the severe decrease in the efficiency of initiation at trpA. This effect is exactly the same what occurs at the trpD-trpE and galK-galT boundaries (Oppenheim and Yanofsky, 1980; Schümperli et al., 1982). We believe that the significance of the results presented

Table 1

Comparison of pALA1 with the frame-shift mutants
pALA1ΔH$_1$ 19, pALA1ΔH$_1$ 30 and pALA1ΔH$_1$ 32 are frame-shift mutant clones derived from pALA1

	β-galactosidase[1] activity	Expressed[2] protein	trp-lac mRNA[3] level	lac mRNA[3] level	lac mRNA half-life time
pALA1	*W*2500	43 Kd β-galactosidase	100%	100%	*W*2.3 min
pALA1ΔH$_1$ 19	96	β-galactosidase	59.5%	41.0%	—
pALA1ΔH$_1$ 30	102	β-galactosidase	69.8%	44.5%	2.0 min
pALA1ΔH$_1$ 32	132	β-galactosidase	71.0%	48.2%	—

[1] β-galactosidase activity is expressed in Miller units.
[2] based on the results of minicell experiments (Fig. 3).
[3] based on the results of hydridization experiments.

here lies in the fact that translational coupling was demonstrated in an artificial operon, where none of the synthetised proteins were identical with the proteins coded by the original operon carrying the boundary. Thus they serve to illustrate that overlapping terminator and initiator codons can be used as portable agents of translational coupling, to ensure high level coordinated synthesis of different proteins coded by genes fused in an artificial operon by recombinant DNA technology.

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Contents

Index: 26.030

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